

**THE ROLE OF PROTEIN SYNTHESIS
IN THE CIRCADIAN CLOCK.
PURIFICATION AND PROPERTIES
OF A POLYPEPTIDE WHICH TENTATIVELY
IS INVOLVED IN THE CLOCK.**

INAUGURAL-DISSERTATION

zur

Erlangung der Doktorwürde

vorgelegt dem Fachbereich Biologie
der Freien Universität Berlin

von

TA-YAN LEONG

aus Ipoh, Malaysia

Mai 1978

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1. Gutachter: Herr Professor Dr. H.-G. Schweiger

2. Gutachter: Herr Professor Dr. H. Clauss

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A. INTRODUCTION

The existence of circadian rhythms in living organisms was first observed when the leaf movement of plants was systematically followed. Since then, detailed characteristics of the circadian rhythm (Biological Clocks, 1960; Bünning, 1973; Hastings and Schweiger, 1976) have been well established even in unicellular organisms such as Acetabularia (Sweeney and Haxo, 1961; Schweiger et al., 1964; Vanden Driessche, 1966; Sweeney et al., 1967; Terborgh and McLeod, 1967), Gonyaulax (Hastings et al., 1961), Chlamydomonas (Bruce, 1970) and Euglena (Pohl, 1948; Bruce and Pittendrigh, 1956; Brinkmann, 1971).

1. Circadian rhythm of photosynthesis and its relationship to chloroplast

Following the development of a sensitive method for continuously measuring over a long period the oxygen output of individual Acetabularia cells, it has become well established that photosynthesis in Acetabularia is characterized by oscillations which have been shown to be circadian in nature (Mergenhagen and Schweiger, 1973; Karakashian and Schweiger, 1976a). In addition to this, it has been shown that the ultrastructure of the chloroplasts of Acetabularia undergoes diurnal changes (Vanden Driessche and Hars, 1972, 1973). Similar circadian rhythms of chloroplast orientation and photosynthetic capacity have been reported in Gonyaulax (Schmitter, 1971; Hermann and Sweeney, 1975) and in Ulva (Britz and Briggs, 1976). The rhythms of chloroplast orientation and that of photosynthetic capacity are probably closely related which ensures maximum efficiency in photosynthesis. Maximum light absorption efficiency is shown to

coincide approximately with maximum photosynthetic capacity in Ulva; suggesting that both parameters may be controlled by the same mechanism. Despite much effort, very little is known about the biochemical basis of the biological clock, largely due to the difficulty of discriminating between a process which is being controlled and the one which is controlling.

By definition, perturbants of the clock would affect the phase of the rhythm, whereas perturbants of processes being controlled by the clock would not bring about permanent phase shift once the perturbants have been removed.

2. Biochemical approach with antibiotics

One experimental approach to investigate the biochemical nature of the biological clock is to use chemicals and metabolic inhibitors to test the effect on the period or phase of a free running circadian rhythm. Depending on the specificity of the treatment, some information may be gained about the nature of the regulatory mechanism. General metabolic inhibitors such as heavy water (D_2O) (Bruce and Pittendrigh, 1960; Bünning and Baltes, 1963); ethanol (Enright, 1971), lithium (Engelmann, 1972) have all been reported to increase the period of the circadian rhythm in at least one organism. By using more selective inhibitors, however, more specific information can be gained. For example, the degree of involvement of genetic information and its expression in the biological rhythm can also be tested by using specific inhibitors.

3. Involvement of gene expression in circadian rhythms

The involvement of genetic information and its expression in the clock has been suggested by the following experiments:-

(a) Nuclear exchange experiments using Acetabularia have shown a direct participation of the cell nucleus in the manifestation of the clock (Schweiger et al., 1964).

(b) Cycloheximide, a specific inhibitor of protein synthesis at the 80S ribosome level, is shown to increase the period of the phototactic response rhythm in Euglena (Feldmann, 1967), and to delay the phase of the photosynthesis rhythm in Acetabularia (Karakashian and Schweiger, 1976a).

(c) Chloramphenicol, a specific inhibitor of protein synthesis at the 70S ribosome level, has been shown to have no effect on the phase and period of the glow rhythm in Gonyaulax (Karakashian and Hastings, 1963) and on the photosynthesis rhythm of Acetabularia (Sweeney et al., 1967; Mergenhagen and Schweiger, 1975; Karakashian and Schweiger, 1976a).

(d) Cycloheximide is shown to inhibit flowering in Xanthium, the inhibition is greatest when the cycloheximide dose is applied either at the beginning or at the end of a 16 hr inductive night (Ross, 1970).

(e) In isolated Aplysia eye, cycloheximide has been reported to cause dose dependent phase delays in the circadian rhythm when administered during projected night at 15° (Rothman and Strumwasser, 1976).

4. Recent studies on Acetabularia with cycloheximide

Following the development of improved procedures for determining and comparing the periods and phases in individual Acetabularia cells (Mergenhagen and Schweiger, 1971; Karakashian and Schweiger, 1976a), it is possible

to thoroughly evaluate the influence of cycloheximide on the autonomous timing systems of Acetabularia. Recent results obtained with this procedure showed that cycloheximide and puromycin pulses delay the phase of the photosynthesis rhythm in individual Acetabularia cells, while chloramphenicol has no effect on the phase of the rhythm, indicating that protein synthesis on 80S ribosomes but not on 70S ribosomes provides a necessary component for the biochemical mechanism of circadian regulation in Acetabularia (Karakashian and Schweiger, 1976b; 1976c).

5. The coupled translation-membrane model

In order to reconcile the biochemical results and the characteristic features of circadian rhythm, a number of models have been proposed; for example the chronon model (Ehret and Trucco, 1967), the network hypothesis (Pavlidis, 1973; 1976; Wagner et al., 1974, 1976), and membrane models (Edmunds and Cirillo, 1974; Njus et al., 1974; Sweeney, 1974). Recent works on Acetabularia have led to the proposal of a "coupled translation-membrane model" (Schweiger and Schweiger, 1977). The two steps under consideration in this model, the synthesis of "essential proteins" on the 80S ribosomes and their integration into membranes are interconnected by a feed-back mechanism. The model implies that the peptide composition of membranes is subjected to diurnal changes. This model is consistent with experimental results derived from the effect of cycloheximide on the photosynthesis rhythm of Acetabularia (Mergenhagen and Schweiger, 1975; Karakashian and Schweiger, 1976b, 1976c).

Cycloheximide treatments have been shown to produce varying effects depending on the temperature and the phase of the

circadian cycle at which the cycloheximide pulse is given. When given during the light phase at 20° , the drug produced no change in phase, whereas a pulse during the light phase at 25° was maximally effective for delaying the phase. Conversely, a pulse given during the dark phase at 20° produced a phase delay of more than 11 hours whereas at 25° , a pulse during the dark phase had no apparent effect on phase.

From these results it was concluded that during distinct phases of the cycle, polypeptides have to be synthesized on 80S ribosomes which are essential for the clock. If their synthesis is inhibited by a cycloheximide pulse the phase will be shifted.

It seems, therefore, feasible to search for the proteins which are essential for the clock. According to the model, the synthesis of these proteins on 80S ribosomes should be sensitive to cycloheximide; the sensitivity towards cycloheximide being temperature and phase dependent. Furthermore, these proteins could be present in the membrane, and a good possibility might be the chloroplast membrane, because of the close relationship of the chloroplast to photosynthesis.

6. Isolation of proteins from the chloroplast membrane

The chloroplasts of Acetabularia are ovoid, more or less elongate organelles which are partially autonomous due to the presence of intrinsic DNA of chloroplastic 70S ribosomes and all the components which are involved in replication, transcription and translation. The whole organelle is enclosed in a double membrane, the constituents of which are synthesized partly in the organelle itself and partly

in the cytosol. The membrane acts as a "gate" to some of the proteins synthesized inside and outside of the organelle.

Although much is known about the structure, function and bioenergetics of the chloroplast (Goodwin, 1971; Arntzen and Briantais, 1975; Avron, 1975; Murakami et al., 1975; Thornber, 1975; Witt, 1975; Combs and Greenwood, 1976; Ellis, 1976; Hooper, 1976; Leech and Murphy, 1976), only a few protein components have been isolated from the chloroplast membrane and characterized (Koenig, 1972; Bengis and Nelson, 1974; Menke et al., 1975; Chua et al., 1975; Apel et al., 1976; Hooper and Stegeman, 1976; Apel, 1977; Bar-Nun et al., 1977), most of them being chlorophyll-protein functional complexes related to the photosystems I and II.

The reasons for the difficulties in isolating protein components from the chloroplast membrane are that the proteins are mostly hydrophobic, as is the case for most other membrane proteins and that the chlorophyll tends to form rather stable complexes with proteins. In most cases, detergents have to be used to bring the proteins into solution (Helenius and Simons, 1975). Once isolated, the proteins tend to aggregate and become extremely difficult to handle, especially when lipids, chlorophyll and detergents are removed. However, recent progress in membrane biochemistry has rendered it possible to investigate the properties and characteristics of hydrophobic membrane proteins in the presence of the detergent sodium dodecyl sulfate (Waehneltdt, 1975; Weber and Osborn, 1975). This has been the most popular detergent in recent studies on chloroplast membrane proteins (Menke et al., 1975).

7. Action of sodium dodecyl sulfate (SDS)

Unlike non-ionic detergents, SDS can be purified by recrystallization. The SDS molecule contains a non-polar hydrophobic chain with a strongly polar anionic end-group and is thus similar in structure to many lipids. It is this similarity to lipids that enables SDS to form a complex with non-polar side chains and charged groups of amino acid residues in polypeptides of all sizes and shapes, without breaking covalent bonds. The binding ratio of SDS to proteins was found to be on a gram to gram basis (Reynolds and Tanford, 1970), although it has also been reported that the primary structure of a protein influences SDS binding. Upon binding of SDS, proteins assume the shape of a rodlike particle, the length of which varies uniquely with the molecular weight of the protein moiety. A complex interplay of net charge, abnormal SDS binding, conformational changes and molecular length may govern the electrophoretic mobility of some proteins.

Thus the multicomponent systems of chloroplast membrane to a certain extent can be dissociated with SDS into their constituent polypeptide chains which can be separated from each other by SDS polyacrylamide gel electrophoresis according to their molecular weights. Proteins of interest can thus be isolated and their properties studied in the presence of SDS or after removal of the detergent.

8. The present approach

The synthesis of proteins in the presence and absence of cycloheximide in Acetabularia can be followed at different temperatures and light-dark phases by incubating cells with radioactive amino acids, isolating different cell fractions

and estimating radioactivity in individual protein components separated by SDS polyacrylamide gel electrophoresis. In this way, as is reported below, there is in particular one polypeptide whose synthesis is strongly inhibited by cycloheximide at 20° in the light phase and at 25° in the dark phase, but is stimulated by the drug at 20° in the dark phase and at 25° in the light phase. This polypeptide was purified by preparative SDS gel electrophoresis and its biochemical nature characterized by performing amino acid analysis, peptide mapping, and amino-terminal analysis.

B. METHODS AND MATERIALS

1. Cells and culture conditions

Cells of Acetabularia mediterranea were cultured in Erd-Schreiber medium (Haemmerling, 1963; Schweiger, 1969) and were entrained to a 12 hour dark and 12 hour light (2500 lux) cycle at 20° or 25°. One week before the beginning of an experiment, 2.0 cm long cells were selected and transferred to fresh medium. Only those cells which had increased their length to 2.5 - 3.0 cm in the week prior to the experiment were chosen for experimental study. Cells were then allowed to free run in constant light (2500 lux), starting from the moment of transfer to new medium.

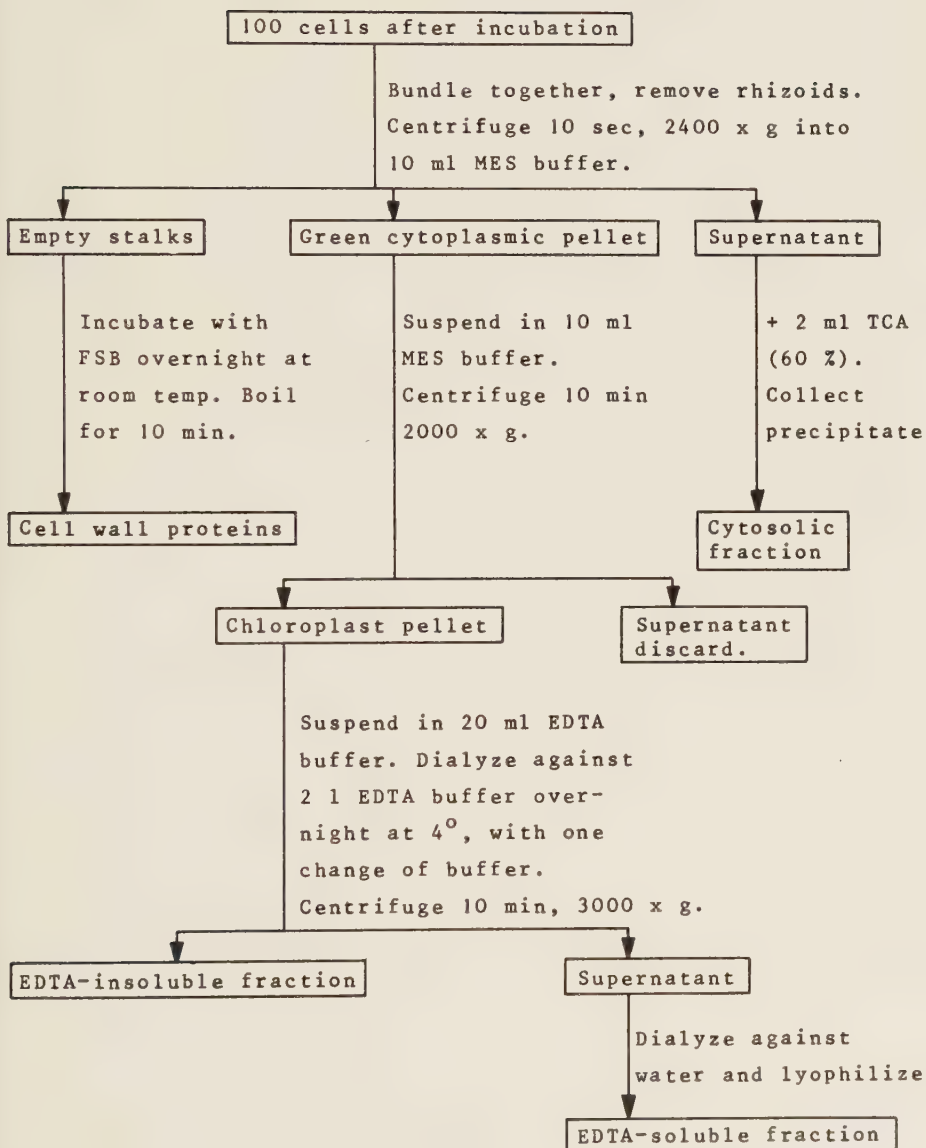
2. Photosynthesis: Incubation in vivo

For routine assays, 100 cells were incubated in 5 ml Erd-Schreiber medium with an illumination of approximately 2500 lux. Preincubation in the presence or absence of cycloheximide (2 µg/ml) began at 4.0 and 16.0 hours circadian times. After 30 min of preincubation, 100 µl (100 µCi) of [³H]amino acid mixture (Amersham Buchler, Code TRK 440, containing the following tritiated amino acids: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine) were added and the incubation allowed to proceed for 3 hours. In other experiments, the tritiated amino acid mixture was replaced by 100 µl (100 µCi) [³⁵S]methionine (Amersham Buchler, Code SJ 204). The cells were then transferred to the chase medium (Erd-Schreiber medium with 0.1 % of an unlabelled protein hydrolysate; Casamino acids, Difco, Detroit) (Apel and Schweiger, 1973) and incubated for

1 hour in the absence of cycloheximide.

3. Fractionation of cells

Immediately after the chase, the cells were bundled together at the apical ends. The rhizoids were removed with a pair of scissors. The bundle was tied to a plastic cover of the centrifuge tube and the cell contents were collected in 10 ml of MES buffer (0.33 M sorbitol, 2 mM NaNO_3 , 2 mM EDTA, 0.5 mM K_2HPO_4 , 20 mM NaCl, 2.5 % Ficoll, 0.01 % bovine serum albumin, 50 mM 2-(N-morpholino)-ethane sulfonic acid, 1 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 1 mM MgCl_2 and 1 mM MnCl_2 , adjusted with 2 N NaOH to pH 6.1) by centrifugation for 10 sec at 2400 x g as described (Schweiger, 1966). Soluble proteins present in the cytoplasm were precipitated by addition of TCA to the supernatant. The precipitate represents the "cytosolic protein fraction". The green pellet obtained after the 2400 x g centrifugation was resuspended in 10 ml MES buffer. The purified chloroplast fraction was obtained by 10 min centrifugation at 2400 x g. The chloroplast fraction was further fractionated after overnight dialysis against the EDTA buffer (0.1 M Tris-HCl, pH 7.6, 1 mM EDTA and 50 mM 2-mercaptoethanol) at 4°. The "EDTA-soluble fraction" was obtained from the dialyzed chloroplast fraction by 10 min centrifugation at 3000 x g, dialysis of the supernatant against water for 24 hours and lyophilization as summarized in the scheme. The "EDTA-insoluble fraction" was prepared from the sediment by washing with 0.1 M Tris-HCl, pH 7.6. All the fractions were dissolved by boiling for 2 min in "Final Sample Buffer" (0.0625 M Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol and 5 % 2-mercaptoethanol) at a concentration of 1 μg chlorophyll per μl FSB; the "cytosolic proteins" and the "EDTA-soluble fraction" were dissolved at a concentration of 1 mg per 100 μl of FSB.



Scheme A. Fractionation of cells.

Electron micrographs of chloroplasts and EDTA-insoluble fraction of the chloroplast obtained in a similar manner have been reported (Apel and Schweiger, 1973).

The empty stalks of the cells were incubated in FSB overnight at room temperature, and finally boiled for 10 min to give a fraction of "cell wall proteins". The amount of total incorporation was determined by digesting the above final samples (10 μ l) in 200 μ l of tissue solubilizer (Soluene 300, Packard) for 30 min at room temperature, and then by adding 10 ml of toluene containing 0.4 % Permablend III (Packard) before measuring by liquid scintillation spectrometry (Packard). The amount of protein was estimated using bovine serum albumin as a standard (Lowry et al., 1951). Chlorophyll content was determined by extracting the EDTA-insoluble fraction with 80 % acetone (Arnon, 1949).

4. Analytical SDS gel electrophoresis

A discontinuous buffer system (Laemmli, 1970) was used in combination with a slab gel apparatus (Studier, 1973). After a series of preliminary electrophoretic runs, the following gel system was found to be most suitable for separating the proteins of interest. The separation gel consisted of two layers, a 15 % gel and a 12.5 % gel (both prepared from the same stock solution of 30 : 0.8 (w/w) of acrylamide to N,N'-methylene-bisacrylamide) in 0.375 M Tris-HCl, pH 8.8,

containing 0.1 % SDS. They were both 4.5 cm long. The stacking gel consisted of a 1 cm layer of a 3 % gel (made from the same stock solution of monomers) and contained 0.125 M Tris-HCl, pH 6.8 and 0.1 % SDS. Polymerization was induced by addition of ammonium persulfate and TEMED (Laemmli, 1970). The 12.5 % gel was carefully layered onto the 15 % gel. SDS (0.1 %) was then layered with a syringe onto the 12.5 % gel and the two layers were allowed to polymerize at the same time. After polymerization of the separation gel (thickness 1 mm), sample wells were formed by allowing the stacking gel to polymerize in the presence of a comb (Studier, 1973). Proteins to be electrophoresed were incubated with the FSB and boiled for 2 min before being applied to the sample wells. The electrode buffer contained 0.025 M Tris-HCl, pH 8.3, 0.192 M glycine and 0.1 % SDS. Electrophoresis was started with a voltage of 70 V (about 35 mA) for an hour and then the voltage was increased to 120 V until the free chlorophyll migrated to the end of the gel (about 5 hours). The gels were stained overnight in a solution containing 110 ml of a 1 % coomassie brilliant blue R-250 (Serva, Heidelberg) solution diluted with 40 ml of acetic acid, 90 ml of water and 200 ml of methanol and destained by diffusion in an aqueous solution of 7 % acetic acid and 10 % methanol. The stained pattern was recorded with a scanner-equipped photometer at 620 nm.

Molecular weights of polypeptides were estimated from a standard plot (Weber and Osborn, 1969) using phosphorylase a (MW = 94,000), bovine serum albumin (68,000), pyruvate kinase

(57,000), ovalbumin (43,000), alcohol dehydrogenase (yeast) (37,000), carbonic anhydrase (29,000), trypsin (23,000), myoglobin (17,200), ribonuclease (13,700) and cytochrome c (11,700). Phosphorylase a, pyruvate kinase, carbonic anhydrase, alcohol dehydrogenase, trypsin and ribonuclease were from Boehringer, Mannheim; bovine serum albumin, ovalbumin, myoglobin, cytochrome c, acrylamide, SDS, TEMED and N,N'-methylenebisacrylamide were supplied by Serva, Heidelberg. Before use, acrylamide, SDS and N,N'-methylenebisacrylamide were recrystallized from chloroform, ethanol and acetone respectively (Ro-Choi et al., 1973).

5. Detection of radioactivity in gels

Radioactivity in the gel was estimated by the following two methods. The gel strips from different assays were laid on a microscope slide and frozen by placing the slide on a metal block which had been precooled in liquid nitrogen. The gel froze in a few minutes and the frozen gel was then sliced into 1 mm thick pieces by pressing with an array of razor blades screwed together with 1 mm spacers separating each and every blade. The frozen gel slices remained sandwiched between the razor blades and were removed individually with a pair of forceps for transfer to vials and treatment with solubilizer (Soluene 300, Packard) for 4 hours at 60°. The vials were then cooled down to room temperature, and to each vial, 10 ml of toluene containing 0.4 % Permablend III (Packard) were added before being counted.

Another quantitative method was to obtain fluorograms of the whole gel (Bonner and Laskey, 1974). The gel was first treated with dimethyl sulfoxide to remove water in the gel, and was then soaked in a 20 % PPO-dimethyl sulfoxide solution. After this treatment, dimethyl sulfoxide was removed by soaking the

gel in water. PPO precipitated and was trapped within the gel. The gel was dried on a piece of Whatman 3 MM filter paper (Maizer, 1971), pressed against an X-ray film (R.P. Royal "X-Omat" film, Kodak), and kept at -70° until developed. This PPO treatment of the gel made it possible to obtain an autoradiogram within a reasonable exposure time from samples which contain tritium as the only source of radioactivity. The autoradiogram was then scanned on a densitometer at 620 nm to estimate the radioactivity in the gel. Both methods of radioactivity detection were employed in this study.

6. Preparative gel electrophoresis (Isolation of polypeptide x)

The EDTA-insoluble chloroplast membrane fraction was subjected to preparative gel electrophoresis (gel: 2.5 mm thick), using the same systems as in analytical gels. The gels were stained with coomassie blue and destained in acetic acid-methanol as described. The band of interest was cut from the gel, rinsed with water and ground with mortar and pestle. Protein was extracted at 50° into several volumes of 0.1 M NH_4HCO_3 containing 0.1 % SDS. The pH was adjusted with NH_4OH to 8.5 - 9 as described (Hoover and Stegeman, 1976). The extraction procedure was repeated for 3 hours or longer. Gel particles were removed by filtration and the extract was lyophilized. The blue powder was dissolved in water and dialyzed against 0.1 % SDS. Coomassie blue was removed from the protein by another preparative SDS gel electrophoresis. Coomassie blue migrated faster than the protein and the blue band of coomassie blue was cut off and discarded. Coomassie blue-free protein was recovered from the rest of the gel by extracting the ground gel with NH_4HCO_3 and 0.1 % SDS as above.

The protein-SDS extract was dialyzed overnight at room temperature against water containing 3 g of Dowex AG 1-X8 (formate form) per 100 ml of water in order to remove SDS. The protein was precipitated by this treatment and collected by centrifugation. The purity of the isolated protein was tested by redissolving it in FSB and reelectrophoresing on an analytical gel. It was also tested for purity by amino-terminal analysis which showed the isolated protein to be of high purity.

7. Amino acid composition

The protein was dissolved in 6 N HCl (including two drops of 5 % phenol) to a final concentration of 1 mg of protein per 4 ml, and sealed under vacuum after the sample had been degassed in the cold for 15 min. Hydrolysis was performed for 24 hours at 110°. The hydrolysate was lyophilized and redissolved in citrate buffer, pH 2.2 to a mg/ml solution and was used for the amino acid composition analysis. The analysis was kindly performed by Dr. K. Apel of the University of Freiburg (Breisgau).

8. Peptide mapping of polypeptide x

Peptide mapping was performed by limited proteolysis in the presence of SDS and the analysis of the fragments by SDS electrophoresis (Cleveland et al., 1977). Purified protein was dissolved at approximately 1 mg/ml in sample buffer which contained 0.125 M Tris-HCl, pH 6.8, 0.5 % SDS, 10 % glycerol and 0.0001 % bromphenol blue. The sample was then heated to 100° for 2 min. Proteolytic digestions were carried out at 37° for 1 hr by addition of 10 μ l of enzyme solutions (mg/ml of sample buffer) to 70 μ l of the protein solutions. Enzyme solutions were prepared from the following: trypsin (TPCK-

treated, from bovine pancreas), α -chymotrypsin, papain (Serva, Heidelberg) and Staphylococcus aureus V8 protease (36-900-1, Miles). Following addition of 2-mercaptoethanol and SDS to final concentrations of 10 % and 2 % respectively, proteolysis was stopped by boiling the samples for 2 min. The peptides were analyzed on a gradient gel of 20 % to 12.5 % with a stacking gel of 8 %. The same buffer systems were used as in analytical gels. Molecular weights of the peptide bands were estimated from a standard plot using peptide fragments obtained by cyanogen bromide fragmentation (Gross, 1967) of cytochrome c and myoglobin (Swank and Munkres, 1971).

9. Amino-Terminal analysis

Amino-terminal analysis was performed with the dansyl chloride method (Gray, 1967), as modified by Weiner (Weiner et al., 1972) and employing 12 nmol protein. The molecular weight of 39,000 as indicated by SDS gel electrophoresis was assumed throughout. The protein (500 μ g) was dissolved in 40 μ l of 10 % SDS, boiled for 2 min and allowed to cool to room temperature. 400 μ l of coupling buffer (0.5 M NaHCO_3 , pH 9.8 with NaOH) and 220 μ l of dansyl chloride (Serva, Heidelberg) (5 mg/ml) in acetone were added. The mixture was incubated for 20 min at 37°, before 200 μ l TCA (60%) were added dropwise. The precipitate was collected by centrifugation, washed once with 1 N HCl to remove dansylic acid. After centrifugation, the supernatant was withdrawn and 100 μ l 6 N HCl were added. A neck was drawn on the tube using a fine oxygen torch, and the tube was sealed under vacuum. After hydrolysis for 18 hr at 105°, the tube was opened and the hydrolysate dried over solid NaOH and concentrated H_2SO_4 in a vacuum desiccator.

The hydrolysis residue was dissolved in 10 μ l of pyridine. Using a μ l capillary, the unknown was repeatedly spotted onto

both sides of the polyamide plate (Schleicher & Schüll, TLC-ready Plastic Sheets F 1700 micro-polyamide plates, coated on both sides without bonding agent, 15 x 15 cm. Article No. 356 000, Lot No.2492) by touching the capillary tip to the polyamide layer. The plate was dried during sample application in a stream of cold air from a hair drier. A standard mixture containing dansylated proline, serine, valine, alanine, glycine, leucine, methionine and asparagine was spotted onto only one side of the plate. The mixture of free amino acids was dansylated by dissolving the mixture in 0.5 ml of 0.1 M NaHCO_3 and adding 0.25 ml of dansyl chloride acetone solution (5 mg/ml). The tube was covered with parafilm and incubated at 37° for 30 min. The reaction was terminated by adding 25 μl of 88 % formic acid. The dansylated standard mixture was used directly for spotting.

Ascending chromatography was performed using the following solvent systems (Hartley, 1970).

Solvent I 1.5 % formic acid in water

Solvent II benzene-acetic acid, 9 : 1

Solvent II was run in the direction perpendicular to Solvent I. As the unknown terminal of polypeptide x could be determined unambiguously after the second dimensional chromatography, no further chromatography in other solvent system was performed.

Chromatogram spots were visualized under ultraviolet light using a mineralogical lamp. Dansylic acid was bright blue, dansyl amide greenish blue and the dansyl amino acids bright yellow. Identification of the unknown was made by comparing both sides of the plate. Ribonuclease, lysozyme, pepsin and myoglobin (Serva, Heidelberg) were used as standard proteins for preliminary trials. Colour positive photographs of the

chromatogram were taken with an overhead ultraviolet lamp on both sides as illuminants; and excess ultraviolet light removed by means of a UV filter before the camera. Black and white prints were then made from the colour positives.

C. RESULTS

1. Temperature and phase dependent effect of cycloheximide

a) Fractionation of chloroplast

Experiments were designed to analyze the electrophoretic pattern of the proteins synthesized by Acetabularia cells at two different phases of the light-dark cycle (0400 - 0730 and 1600 - 1930, CT), starting 4 hours after the beginning of the two phases. Reportedly, there is extensive growth of thylakoid membranes and association of 70S ribosomes with thylakoid membranes at the 4th - 5th hour of the light phase (Chua et al., 1976). On the other hand, there is no binding of 70S ribosomes to membranes during the dark phase of the cell cycle when there is no growth of thylakoid membranes in Chlamydomonas (Schor et al., 1970). Both the light and dark phase experiments were performed at two temperatures; namely, at 20° and 25°. These combinations of time and temperature were chosen also because previous experiments (Karakashian and Schweiger, 1976c) had revealed that these conditions produced maximum and minimum phase shifting responses when the cells were subjected to low concentrations of cycloheximide. Under the conditions of 20° at 0600 and 25° at 1800, no phase shifting was observed in the presence of cycloheximide while at 1800 at 20° and 0600 at 25°, the inhibitor resulted in a maximum phase shifting of up to 12 hours.

Under each of these four conditions, 100 cells were incubated as controls, and 100 were treated with a three hour pulse of cycloheximide, i.e., there were eight sets of 100 cells each. In order to minimize any difference among cells resulting from a direct response to light, they were

allowed to free run prior to incubation in constant light while the drug and labelled amino acids were being administered.

Samples treated with cycloheximide were preincubated in the presence of the drug 30 min before beginning of the three hour incubation in the presence of both the labelled amino acids and cycloheximide. At the end of the three hour labelling period, the samples were transferred into cycloheximide-free medium and chased for one hour with an unlabelled amino acid mixture.

The cells were then harvested and divided into four cell fractions; the cytosolic proteins, a chloroplast fraction soluble in EDTA buffer, a chloroplast fraction insoluble in EDTA buffer and a fraction that was obtained by treating the stalks with SDS after the contents of the cytoplasm had been removed by centrifugation (see METHODS AND MATERIALS and Scheme A). These four fractions were obtained from each of the eight samples, dissolved in SDS and run on slab polyacrylamide gel electrophoresis. Only the results of the EDTA-insoluble chloroplast membrane fraction are presented here since that was the only fraction in which a correlation was observed between the temperature and phase dependent effect of cycloheximide on the profiles of incorporation and the biological observations of cycloheximide sensitivity/insensitivity (Karakashian and Schweiger, 1976c).

b) Analytical gel electrophoresis

A coomassie blue stained analytical gel on which the EDTA-insoluble chloroplast membrane fraction had been separated by SDS gel electrophoresis was subjected to densitometric scan at 620 nm (Fig. 1). At least 40 bands can be detected, pointing to the complex nature of this fraction. The staining pattern is essentially indistinguishable between assays.



Fig. 1. Densitometric scanning at 620 nm of a stained gel after electrophoretic separation of the EDTA-insoluble chloroplast fraction. Positions of standard proteins are inserted on top ($MW \times 10^{-3}$).

The prominent bands corresponding to molecular weights of 24,000 and 22,500 probably can be assigned to the two sub-units of the photosystem II complex (Apel, 1977). The overall polypeptide profile is similar to the patterns reported for the thylakoid membranes of higher plants and other algae (Eaglesham and Ellis, 1974; Thornber, 1975; Hooper and Stegeman, 1976).

c) Incorporation of [^3H]amino acid mixture into the EDTA-insoluble chloroplast fraction

A set of incorporation experiments was performed and the radioactivity is plotted against migration distance (Fig. 2). The patterns of incorporation under the four variations of phases and temperatures are compared between controls (solid lines) and after cycloheximide treatment (broken lines). If cycloheximide is added at 20° in the light phase (2 $\mu\text{g/ml}$), it inhibits incorporation of labelled amino acids into almost all polypeptides (Fig. 2a). The pattern at 25° in the light phase is quite different (Fig. 2b), although incorporation into the fractions 52 and 54 is inhibited by cycloheximide, most of the other polypeptides are not affected. In fact, incorporation into the region of fractions 30 through 40 is even stimulated by cycloheximide. At 20° in the dark phase, this stimulating effect of cycloheximide becomes more pronounced (Fig. 2c). Under these conditions, incorporation into fraction numbers 33 and 38 is strongly

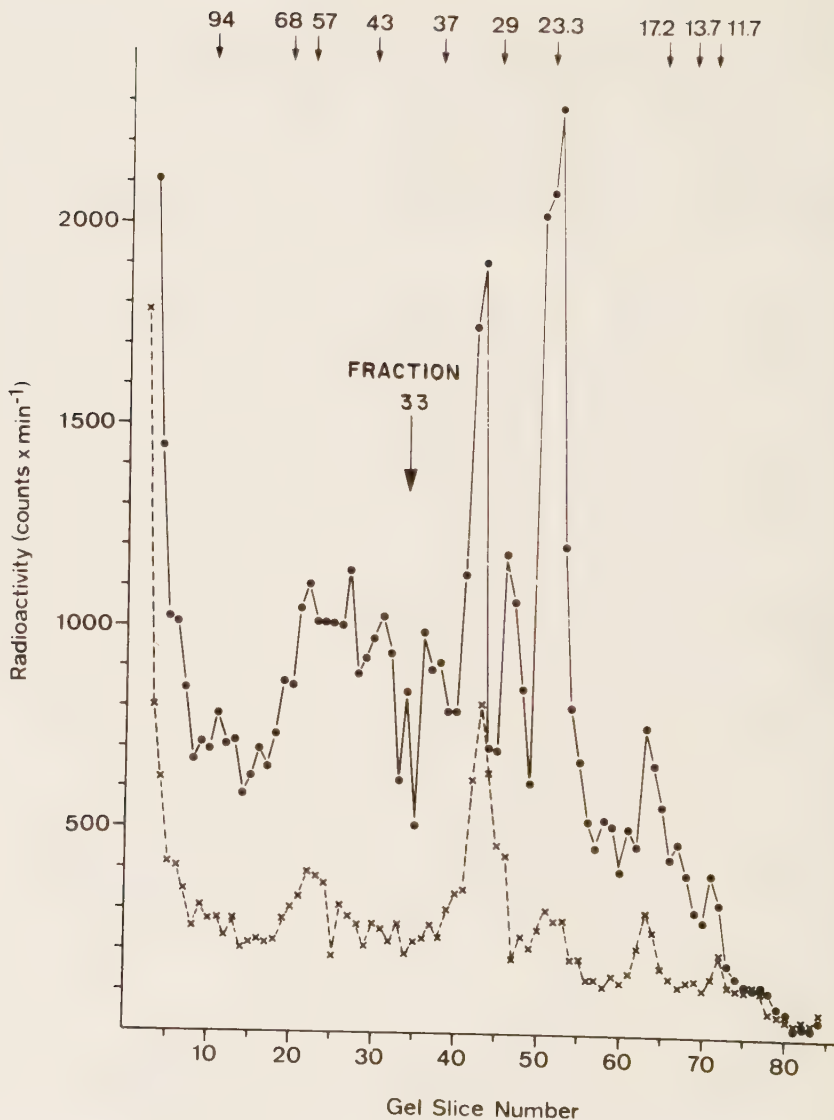
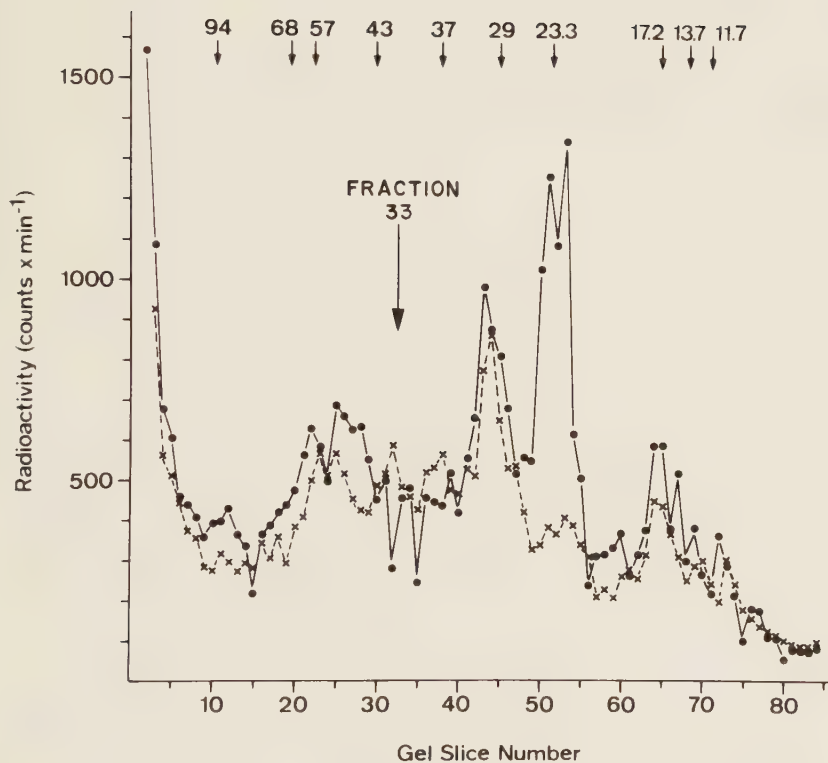
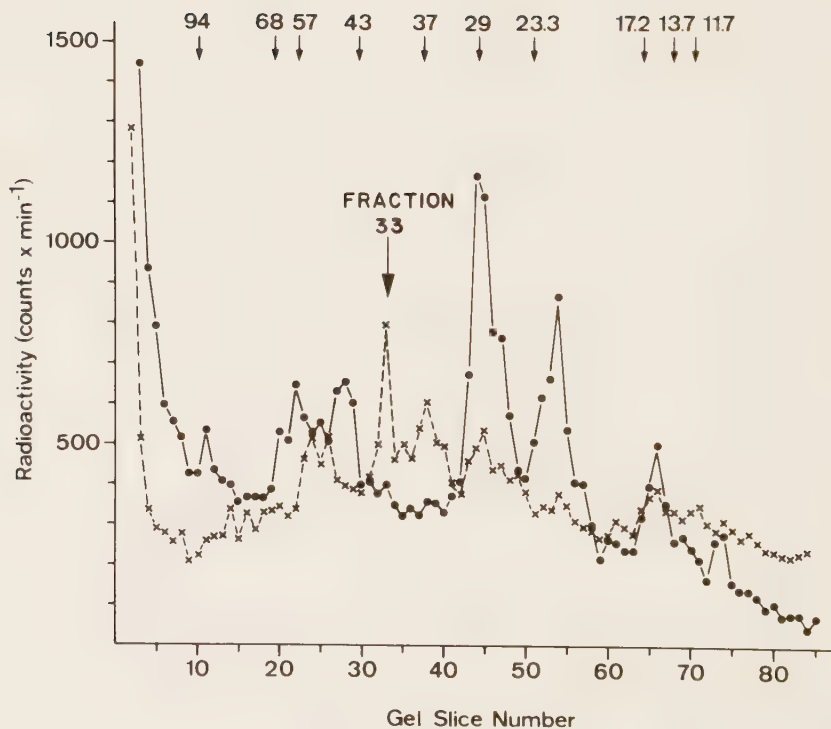


Fig. 2. Incorporation of [³H] amino acid mixture into EDTA-insoluble chloroplast fraction. ·-·, control; x-x, in the presence of cycloheximide. Positions of standard proteins inserted on top (MW x 10⁻³).

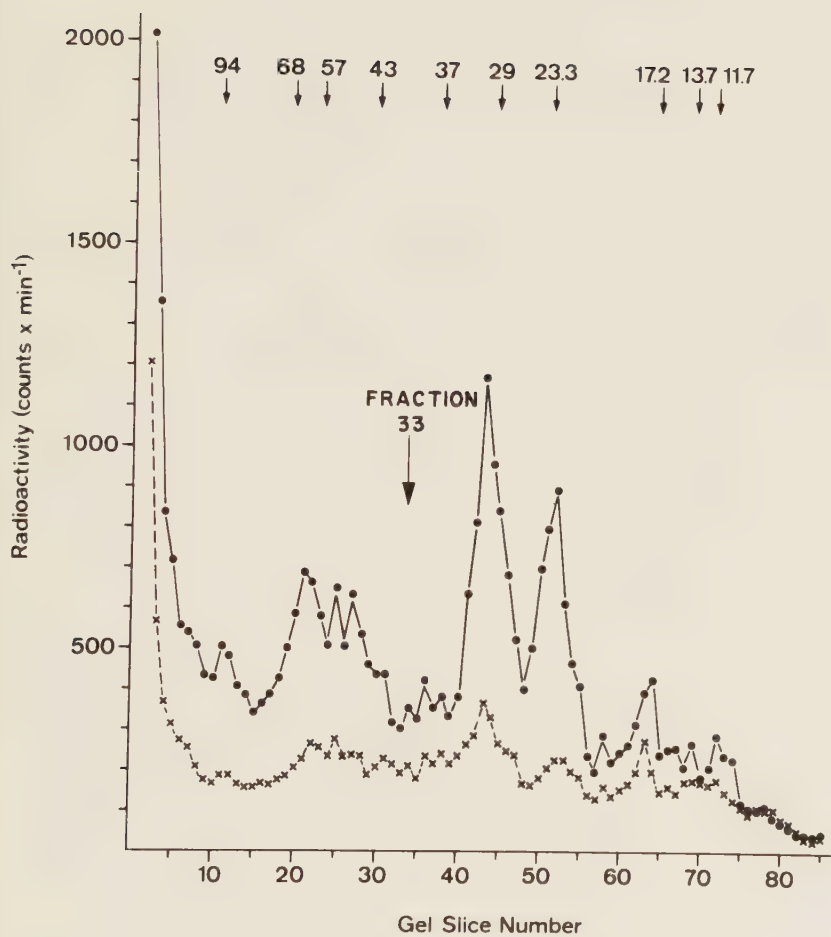
2a) Incubation at 20°, light phase.



2b) Incubation at 25°, light phase.



2c) Incubation at 20⁰, dark phase.



2d) Incubation at 25°, dark phase.

enhanced in the presence of cycloheximide while incorporation into other polypeptides is reduced. However, at 25° in the dark phase, incorporation into all polypeptides is strongly inhibited by cycloheximide (Fig. 2d). It is therefore clear that the effect of cycloheximide is rather different under varying combinations of temperatures and times of the light-dark phases. The two peaks corresponding to molecular weights of 24,000 and 22,500 (slice numbers 51 and 53) may be assigned as the two sub-units of the photosystem II complex (Apel, 1977). From the patterns of incorporation, it is obvious that these two proteins are synthesized preferentially in the light phase and they are inhibited by cycloheximide under all conditions of incubation studied. This result is consistent with a previous study (Machold and Aurich, 1972).

The polypeptide which most significantly exhibits the temperature and phase dependent effect of cycloheximide is fraction number 33 in Figs. 2b and 2c. It has an approximate molecular weight of 39,000 (see Section g) and is termed polypeptide x in this study.

Thus it becomes clear that the appearance/synthesis of polypeptide x is inhibited by cycloheximide when the drug is applied at 20°, light phase; and at 25°, dark phase, whereas its appearance/synthesis is stimulated by cycloheximide at 20°, dark phase and at 25°, light phase.

d) Comparison of the two methods of detection of radioactivity in gels

The same set of experiments was analyzed in such a way that the radioactivity in the gel was detected by the method of fluorography (METHODS AND MATERIALS). Although the densitometric scanning at 620 nm does not measure quantitatively

the radioactivity in the gel, it does allow qualitative comparison among assays. Furthermore, it offers the advantage of being precise in that even closely spaced bands can be detected, whereas in the gel slice method, it is impossible to resolve individual bands which are within a 1 mm slice since only the total radioactivity of all bands in that slice is counted. In addition, the fluorogram renders it possible to have all the assays on the same film, making it easier to detect and compare even minor differences. When tritium is the isotope present in the gel, it is faster to use the gel slicing method to measure the radioactivity in the gel as the fluorography method takes a longer time of exposure, even though the gel has been treated with PPO. Both methods have been used in order to minimize any ambiguity in detecting radioactivity in the gels, and both gave essentially identical results (Figs. 3a - 3h, position of polypeptide x indicated by arrow). It is obvious that the treatment with cycloheximide has different inhibiting and/or stimulating effects depending on the time of application and temperature. When applied during the light phase at 20° (Fig. 3b) or during the dark phase at 25° (Fig. 3h), cycloheximide inhibits incorporation into almost all polypeptides. However, when applied during the light phase at 25° (Fig. 3d) or during the dark phase at 20° (Fig. 3f), cycloheximide inhibits incorporation into some polypeptides and stimulates incorporation into other polypeptides.

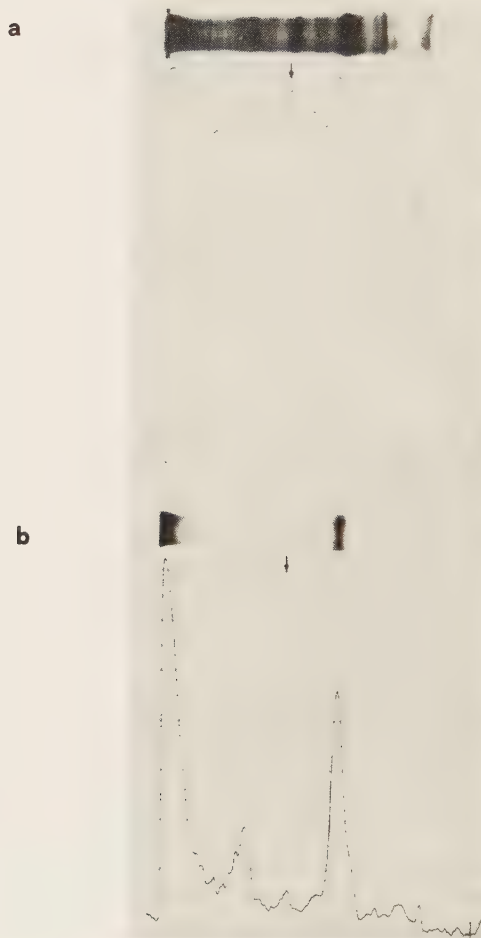


Fig. 3. Incorporation of [^3H]amino acid mixture into EDTA-insoluble chloroplast fraction, showing autoradiograph and scanning (620 nm).

3a) Incubation at 20° , light phase, control.

3b) Incubation at 20° , light phase, in the presence of cycloheximide.

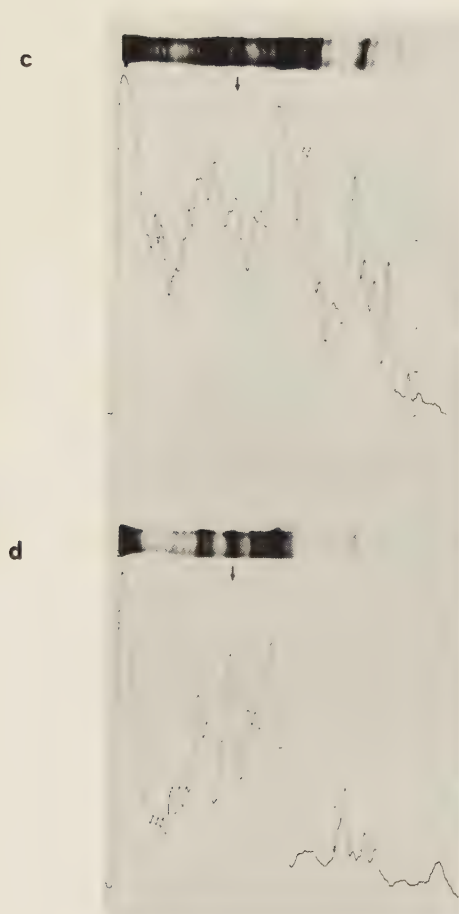


Fig. 3c) Incubation at 25°, light phase, control.

3d) Incubation at 25°, light phase, in the presence of cycloheximide.

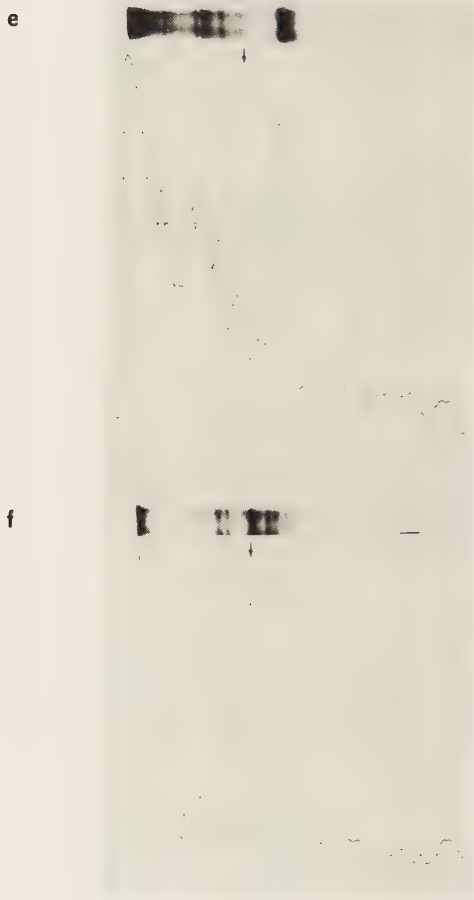


Fig. 3e) Incubation at 20⁰, dark phase, control.

3f) Incubation at 20⁰, dark phase, in the presence of cycloheximide.

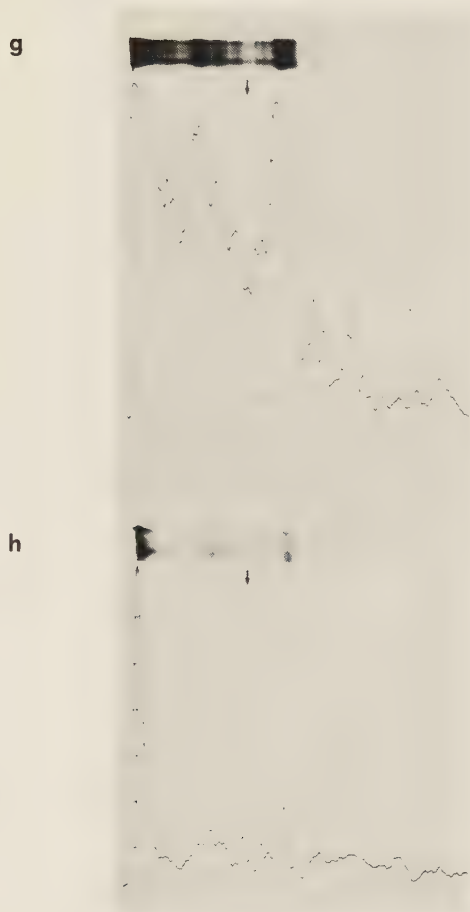


Fig. 3g) Incubation at 25⁰, dark phase, control.

3h) Incubation at 25⁰, dark phase, in the presence of cycloheximide.

The results show that the effect of cycloheximide is temperature and phase dependent. Furthermore, it becomes clear that both the gel slicing method and the fluorography method are suitable for detecting the radioactivity patterns in the gel.

e) Incorporation of [^{35}S]methionine into EDTA-insoluble chloroplast fraction

In order to eliminate any doubt as to whether the radioactivity detected in the gels reflects protein synthesis as opposed to micellar forms or aggregates of the radioactive amino acids, another set of experiments was performed with [^{35}S]methionine as the radioactive precursor. Since the earlier results were obtained from incubations using a methionine-free tritiated amino acid mixture, results obtained from incubation with [^{35}S]methionine would offer a good comparison, although special attention should be paid during interpretation of results with [^{35}S]methionine to the possibility that the radioactivity detected might reflect only the abundance of methionine in the polypeptide and might not present a fair picture of the synthesis of the polypeptide.

The experiments with [^{35}S]methionine were evaluated by means of autoradiographs which were obtained from a 10 day exposure of the X-ray film to the PPO treated gel at -70° , and the densitometric scanning (620 nm) of this autoradiograph (Fig. 4a - 4h, position of polypeptide x indicated by arrow). Once again, the temperature and phase depending effects of cycloheximide are evident. The results support the results from methionine-free amino acid mixture incubations (Figs. 2 and 3): (a) incorporation into polypeptide x is stimulated

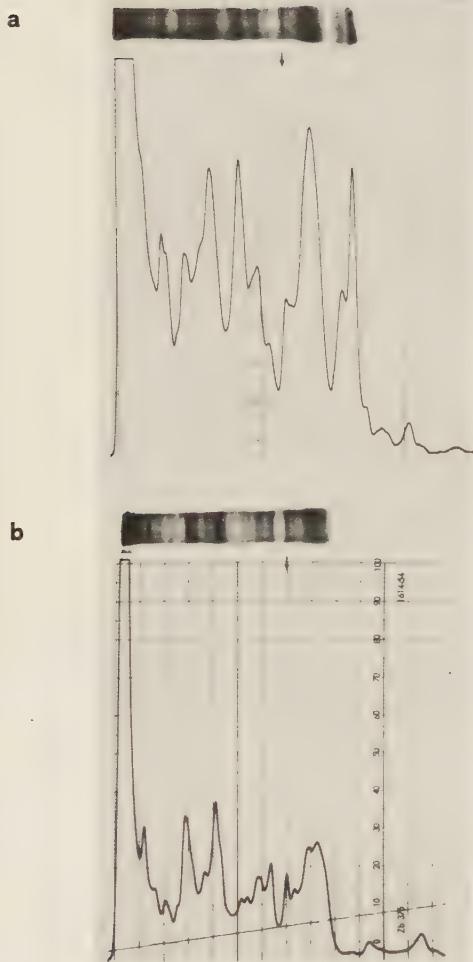


Fig. 4. Incorporation of [^{35}S]methionine into EDTA-insoluble chloroplast fraction, showing autoradiograph and scanning.

4a) Incubation at 20 $^{\circ}$, light phase, control.

4b) Incubation at 20 $^{\circ}$, light phase, in the presence of cycloheximide.

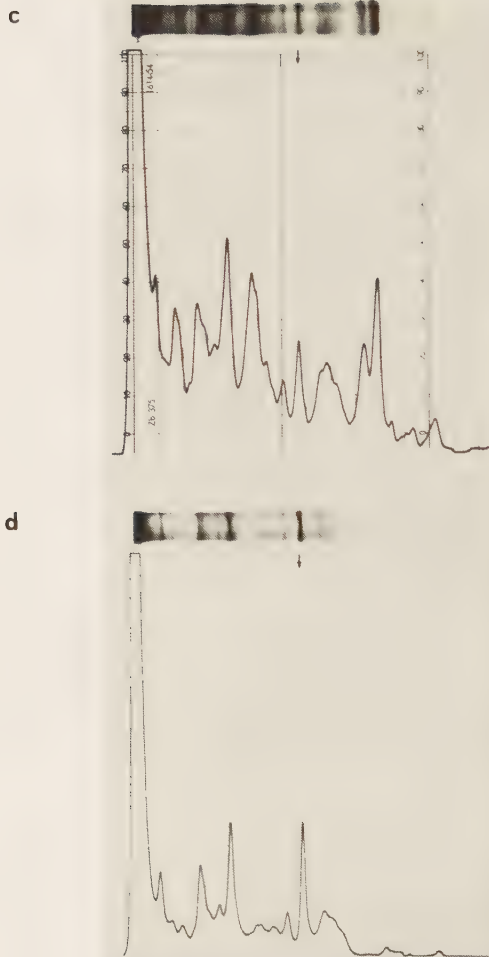
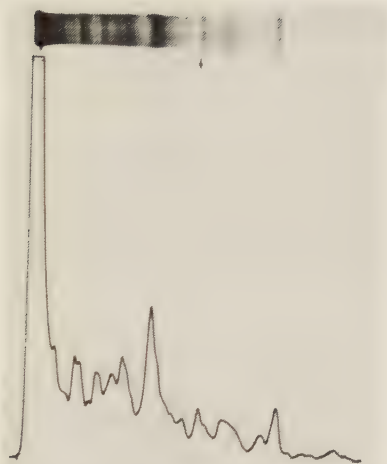


Fig. 4c) Incubation at 25⁰, light phase, control.

4d) Incubation at 25⁰, light phase, in the presence of cycloheximide.

e



f

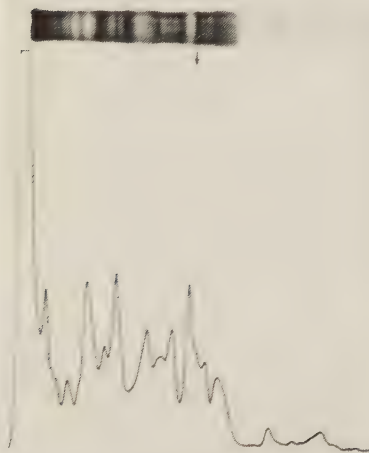
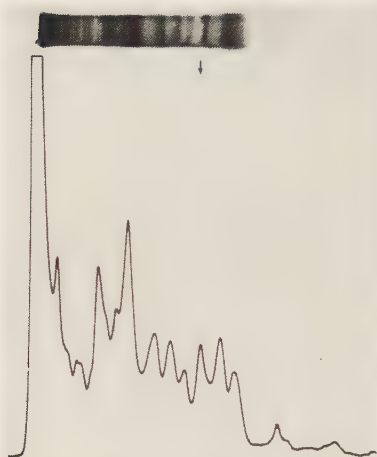


Fig. 4e) Incubation at 20° , dark phase, control.

4f) Incubation at 20° , dark phase, in the presence of cycloheximide.

g



h

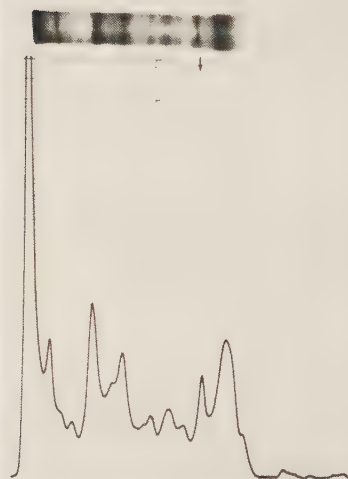


Fig. 4g) Incubation at 25° , dark phase, control.

4h) Incubation at 25° , dark phase, in the presence of cycloheximide.

by cycloheximide during the light phase at 25° (Fig. 4d) and during the dark phase at 20° (Fig. 4f); and (b) incorporation into the two sub-units of the photosystem II complex (polypeptides having molecular weights of 22,500 and 24,000) proceeds preferentially in the light phase but is always inhibited by cycloheximide.

f) Incorporation into other chloroplast proteins

The effect of cycloheximide on the incorporation of labelled amino acids into EDTA-insoluble chloroplast proteins is in different ways temperature and phase dependent (Tab. 1 and Tab. 2). Two different types of polypeptides are represented by polypeptide x which seems to be an exception (Tab. 1), and by the 24,000 polypeptide sub-unit of the photosystem II complex which reflects the general behaviour (Tab. 2).

g) Molecular weight of polypeptide x

Products whose synthesis exhibits a temperature and phase dependent sensitivity to cycloheximide, e.g. polypeptide x, have thus become the center of interest in the present study. First of all, the electrophoretic behaviour of polypeptide x was compared with that of standard proteins of known molecular weight in order to determine the approximate molecular weight of polypeptide x. The electrophoretic mobilities of standard proteins were determined from the stained gel using free chlorophyll as a reference marker, instead of dye markers which are lost after destaining (Weber and Osborn, 1969). The values of electrophoretic mobilities were then plotted against the logarithms of the molecular weight of the corresponding proteins, giving a straight line in the range of 94,000 to 11,700 (Fig. 5). The electrophoretic mobility of polypeptide x

Table 1. Labelling of polypeptide x under different conditions. Radioactivity was estimated from a scan at 620 nm of X-ray films exposed to gels which had been treated with PPO as described under "METHODS AND MATERIALS". Values for the height (in arbitrary units) of each peak are the averages from two experiments.

Source of isotope	Temperature	Condition	Relative incorporation into polypeptide x	
			Light phase	Dark phase
^{35}S	20°	- CX	68	24
	20°	+ CX (2 $\mu\text{g}/\text{ml}$)	36	71
	25°	- CX	49	47
	25°	+ CX (2 $\mu\text{g}/\text{ml}$)	59	41
^3H	20°	- CX	186	66
	20°	+ CX (2 $\mu\text{g}/\text{ml}$)	24	147
	25°	- CX	109	105
	25°	+ CX (2 $\mu\text{g}/\text{ml}$)	137	11

Table 2. Characteristic labelling of an EDTA-insoluble chloroplast membrane protein (MW = 24,000). Conditions are the same as in Table 1.

Source of Isotope	Temperature	Conditions	Relative incorporation into polypeptide	
			Light phase	Dark phase
^{35}S	20°	- CX	73	12
	20°	+ CX (2 $\mu\text{g/ml}$)	14	3
	25°	- CX	48	16
	25°	+ CX (2 $\mu\text{g/ml}$)	12	7
^3H	20°	- CX	165	30
	20°	+ CX (2 $\mu\text{g/ml}$)	10	11
	25°	- CX	146	60
	25°	+ CX (2 $\mu\text{g/ml}$)	18	13

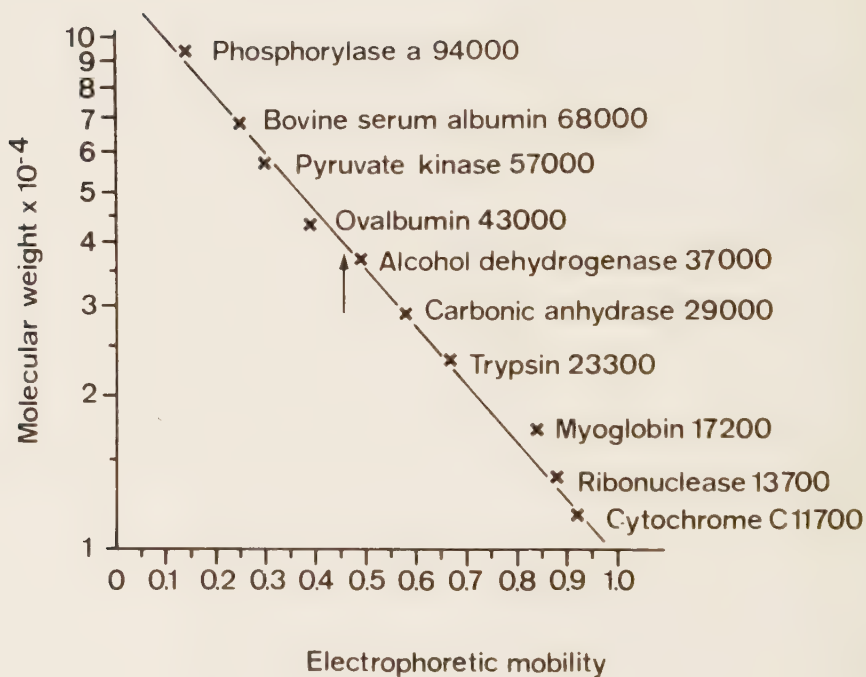


Fig. 5. Electrophoretic behaviour of standard proteins and polypeptide x (arrow). Numbers following the proteins give molecular weights.

with reference to the free chlorophyll was plotted onto the calibration curve and gives a molecular weight of 39,000. This value is only reliable when the binding of SDS to the molecule is normal and the electrophoretic mobility is not complicated by other factors. The MW of 39,000 is supported by amino acid composition and peptide mapping.

2. Isolation of polypeptide x

In order to further characterize polypeptide x, it was necessary to isolate the polypeptide in a reasonably pure state and in a sufficient amount. Since the analytical gel electrophoresis gave good separation of the chloroplast fraction, the whole gel system of the analytical gel electrophoresis was scaled up for preparative electrophoresis. The thickness of the gel was increased to 2.5 mm and the EDTA-insoluble chloroplast fraction was applied onto the whole flat surface of the stacking gel. Overloading was avoided in order to maintain the good separation capability of the analytical gel.

The preparative gels were stained and destained so that only the band of interest was removed, without contamination by fine bands in the neighbourhood. The coomassie blue was later removed by taking advantage of the fact that the dye migrates faster than the polypeptide in an SDS gel. The SDS bound to the isolated polypeptide was removed by dialyzing the polypeptide-SDS solution against a suspension of Dowex AG 1-X8. The solution was not allowed to come into direct contact with the ion-exchanger (Weber and Kuter, 1971) in order to prevent absorption of the protein onto the ion-exchanger.

The purity of the isolated polypeptide was checked by reelectrophoresis on an analytical gel (Figs. 6 and 7). The isolated polypeptide seems to be very pure as there is only one band in the reelectrophoresis, suggesting that there was no aggregation or dissociation during purification (Fig. 6). Densitometric scanning of the stained gel supports this fact (Fig. 7). However, homogeneity on an SDS gel electrophoresis does not necessarily mean that the isolated polypeptide is free from contamination since polypeptides of the same molecular weight or polypeptides having a molecular weight difference of a few hundred and proteins of similar electrophoretic mobility would not be separated and would migrate to the same place under this condition. Hence, the purity of the isolated polypeptide x was further tested by performing amino-terminal analysis, which gives very strong evidence for the purity of the polypeptide (see Section 3d).

3. Characteristics of polypeptide x

a) Amino acid composition

The amino acid composition of polypeptide x was determined (Tab. 3), and the amino acids were arranged into hydrophilic, intermediate and hydrophobic groups (Steck and Fox, 1972). The analysis shows that the proportion of non polar intermediate amino acid residues is rather high, as expected for membrane proteins. Furthermore, the content of hydrophilic amino acids is well balanced by almost the same proportion of hydrophobic amino acid. The proportion of proline is significantly low while that of glycine is significantly high. From the estimated number of residues per molecule, the molecular weight of polypeptide x is calculated to be approximately 39,700.

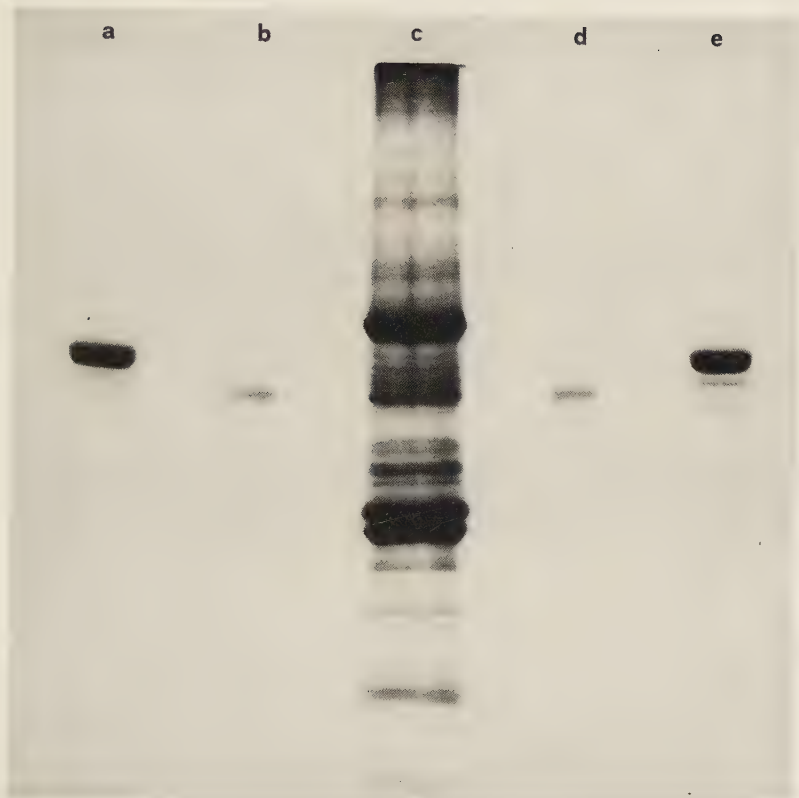


Fig. 6. Reelectrophoresis of polypeptide x, showing staining pattern of (a) ovalbumin, (b) polypeptide x, (c) EDTA-insoluble chloroplast fraction, (d) polypeptide x, and (e) ovalbumin.



Fig. 7. Reelectrophoresis of polypeptide x, showing densitometric scanning of gel (b) in Fig. 6. (620 nm).

Table 3. Amino acid composition of polypeptide x and other chloroplast components

	Poly-peptide x		Estimated number of residues per molecule		67,000 chlorophyll-protein		21,500 subunits of photosystem II complex		23,000 chlorophyll-protein		125,000 chlorophyll-protein	
	mol %				mol % ⁺		mol % ⁺		mol % ⁺		mol % ⁺	
Asx	8.4		27		9.3		9.3		9.1		8.1	
Glx	6.8		22		9.6		8.5		9.3		6.7	
His	4.5		15		1.1		0.7		1.1		4.0	
Lys	7.5		25		4.2		4.7		4.6		7.8	
Arg	4.1		13		3.3		3.0		3.2		6.4	
Thr	4.5		15		4.6		4.4		4.5		5.1	
Ser	4.5		15		6.2		6.0		5.8		7.4	
Pro	1.6		5		6.4		7.0		7.3		3.8	
Ala	7.9		26		9.6		9.0		9.6		9.3	
Cys	-		-		-		-		-		-	
Gly	16.7		55		15.7		16.0		15.6		9.8	
Tyr	3.6		12		3.5		3.9		4.2		2.9	
Val	6.6		22		5.7		6.0		5.3		6.7	
Ile	7.2		24		4.6		4.6		4.5		5.7	
Leu	8.6		28		9.3		9.5		9.1		9.7	
Phe	3.4		11		5.7		6.0		5.7		6.2	
Met	1.8		6		1.3		1.8		1.4		1.1	
hydrophilic		31.3 %	Estimated									
intermediate		35.2 %	MW = 39,703									
hydrophobic		31.2 %										

⁺Values taken from Apel (1977)

b) Comparison with other chloroplast membrane proteins

The amino acid composition of polypeptide x was compared with those of the photosystem I (MW = 125,000) and photosystem II (MW = 67,000) complexes and the two sub-units of the photosystem II complex (the 21,500 and the 23,000 sub-units) of Acetabularia (Apel, 1977) (Tab. 3). The high proportion of glycine is also observed in all components compared except the 125,000 chlorophyll-protein complex. The content of phenylalanine, alanine, proline and serine in polypeptide x is rather low when compared with that of other Acetabularia chloroplast proteins while the content of isoleucine in polypeptide x is higher than that in the other proteins. It is of interest to note that the proportion of hydrophilic amino acid groups in polypeptide x is significantly higher, whereas that of the intermediate amino acid groups is significantly lower than those of the 67,000 chlorophyll-protein complex and the two sub-units. It is clear that although there are some similarities in the proportions of some amino acids, the amino acid composition of polypeptide x is different from that of other Acetabularia chloroplast membrane proteins.

c) Peptide mapping

Because of the difficulty of isolating large amounts of polypeptide x, the conventional method of electrophoresis and chromatography on thin layer plates was not employed. Instead, a new method (Cleveland et al., 1977) was adopted, which offers the following advantages:- proteolysis is performed in the presence of SDS, so that material isolated from SDS gels can be used directly and thereby avoiding the loss arising during SDS removal; the peptides obtained after proteolysis with different enzymes are separated on the same gel, making accurate comparison possible; when the analysis

of peptides is performed together with standard peptides of known molecular weight, the molecular weight of the peptides of interest can be determined and this new method is much more sensitive because detection of peptides is by coomassie blue staining as compared to the less sensitive ninhydrin detection of peptides on conventional thin layer plates.

Proteolysis in SDS was performed with chymotrypsin, trypsin, papain and Staphylococcus aureus V8 protease (Fig. 8). Peptide fragments from cyanogen bromide treatment of cytochrome c and myoglobin served as standard peptides for molecular weight determination. The electrophoretic mobilities of the different peptide fragments of known molecular weight were plotted against logarithms of the molecular weight (Fig. 9). Instead of having one straight line for the standard plot, two straight lines were obtained, intercepting at about 11,000, i.e. one straight line is for calibrating peptides of molecular weight larger than 11,000 and the other is for peptides between 11,000 and 1,500. This phenomenon has been observed in other gel systems (Swank and Munkres, 1971).

Using the above calibration curve, the molecular weights of peptides obtained after different enzyme digestions of polypeptide x were determined (Tab. 4). The molecular weights of the different peptide fragments add up to approximately 39,100, which is very close to the value of 39,000 obtained for intact polypeptide x in the previous SDS gel electrophoresis (Section 1 g). It is therefore reasonable to conclude that the molecular weight of polypeptide x is approximately 39,000.

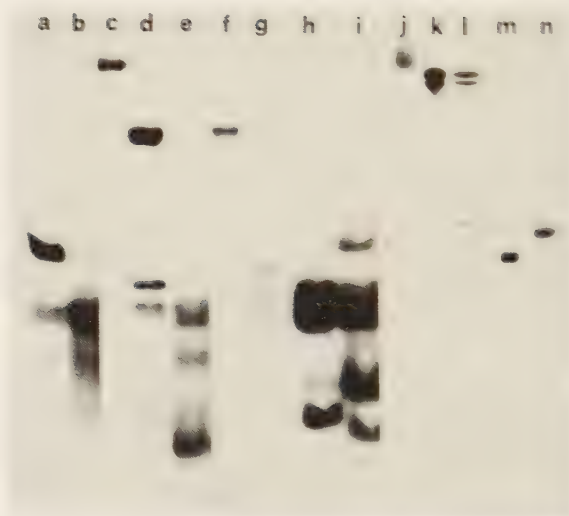


Fig. 6. Peptide mapping of polypeptide 7.

- a) Chymotrypsin
- b) Digestion with chymotrypsin
- c) Undigested polypeptide 7
- d) Trypsin
- e) Digestion with trypsin
- f) Sapaia
- g) Digestion with sapaia
- h) CNBr treated cytochrome c
- i) CNBr treated myoglobin
- j) Undigested polypeptide 7
- k) V8 protease
- l) Digestion with V8 protease
- m) Cytochrome c
- n) Myoglobin

a) - 1) separated on a 20 - 12.5% gel.

l) - 2) separated on a 15% gel.

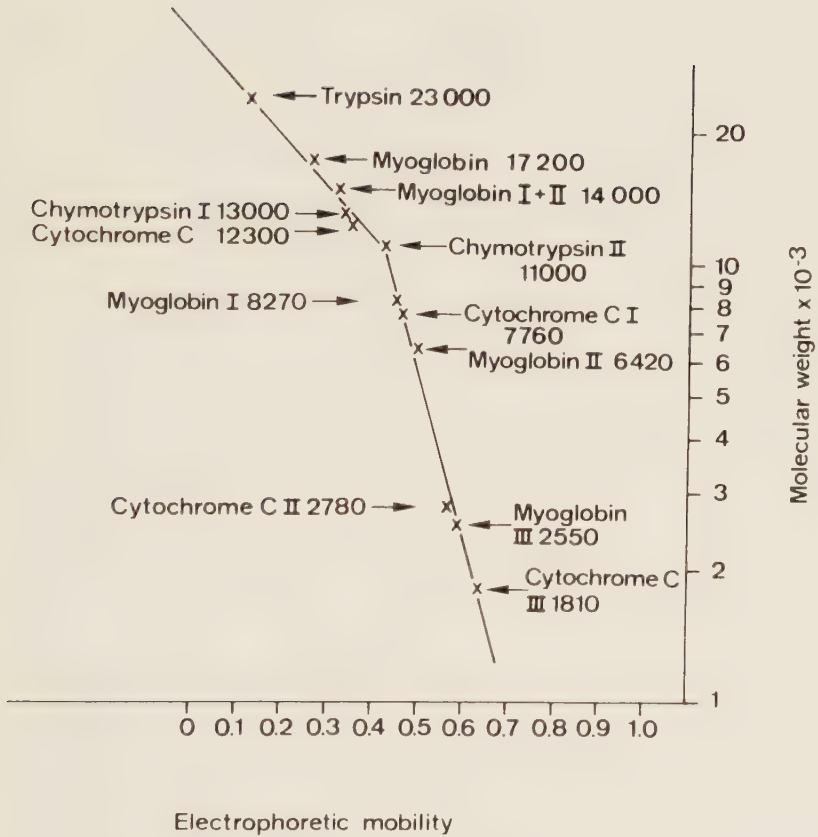


Fig. 9. Electrophoretic behaviour of standard peptide fragments.

Numbers give molecular weights of peptides.

Table 4. Peptide mapping of polypeptide x

[illegible]

* Peptide fragments separated on 20 - 12.5 % gel.

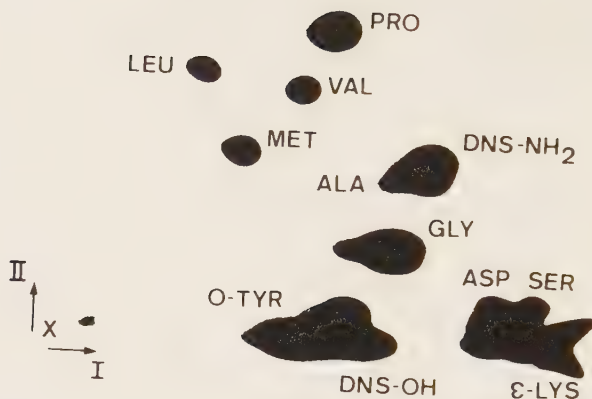
****** Peptide fragments separated on 15 % gel.

*** The first peptide (MW = 30,000) was considered as the aggregate form of the next two peptides (MW = 17,900 and 12,100).

d) Amino-terminal analysis

The modified method (Weiner et al., 1972) adopted for dansylating proteins in the presence of SDS was employed so as to avoid the step of SDS removal from isolated polypeptide x. Polyamide plates coated on both sides were employed and the unknown material was spotted on both sides, while only one side of the plate received also a mixture of standard dansylated amino acids. After chromatography, only the unknown material is on one side and the unknown material together with the standard amino acid mixture on the other side. This facilitates identification and assignment of amino acid groups to the unknown material, and furthermore when the unknown material coincides with one of the standard amino acids, this would give a spot stronger than the other amino acids. This was indeed the case (Fig. 10a). When 8 nmol of dansylated polypeptide x were spotted alone, a rather big spot above the o-tyrosine spot was detected besides the spots of ϵ -lysine, o-tyrosine and dansylic acid which are always present with dansylated proteins (Fig. 10b). This spot was assigned as dansylated glycine after comparing with the chromatogram of 4 nmol of dansylated polypeptide x together with the standard dansylated amino acid mixture which contained 2 nmol each of dansylated proline, valine, leucine, methionine, alanine, asparagine, serine, glycine and ammonia (Fig. 10a). The glycine spot in the chromatogram which contained polypeptide x plus amino acid mixture (Fig. 10a) was bigger than in the chromatogram which contains only the standard dansylated amino acid mixture (Fig. 10 c). The glycine spot (Fig. 10 c) was identified as such by comparison with a chromatogram of dansylated glycine (4 nmol) spotted onto the opposite of the plate (Fig. 10d). Thus, the amino-terminal amino acid of polypeptide x is unambiguously assigned as glycine.

a



b

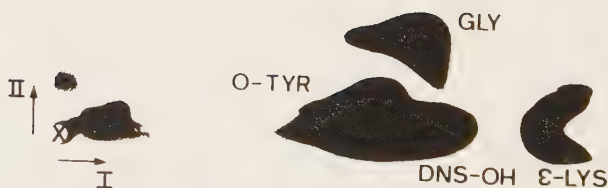


Fig. 10. Two-dimensional thin layer chromatographs.

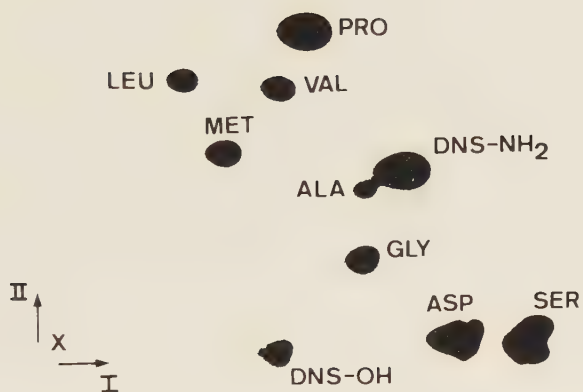
DNS-OH: 1-dimethyaminonaphthalene-5-sulfonic acid,

DNS-NH₂: 1-dimethyaminonaphthalene-5-sulfonamide.

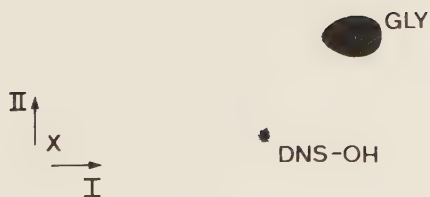
10a) Dansylated polypeptide x and standard amino acid mixture.

10b) Dansylated polypeptide x alone.

c



d



10 c) Dansylated standard amino acid mixture.

10 d) Dansylated glycine alone.

The amino-terminal analysis further supports the claim that the isolated polypeptide x is very pure. Only the one spot of dansylated glycine could be detected (Fig. 10b), although there were some minor spots at the starting point. It seems that the preparation of isolated polypeptide x is not greatly contaminated by other peptides which have similar molecular weights or similar electrophoretic mobilities. The possibility that polypeptide x was contaminated with other polypeptides having similar molecular weights, similar electrophoretic mobilities and the same N-terminal amino acid is very unlikely.

D. DISCUSSION

1. Introduction

On the basis of the "coupled translation-membrane model", it has been predicted that during distinct phases of the circadian cycle (light phase at 25° and dark phase at 20°) polypeptides which are essential to the clock would be synthesized on 80S ribosomes. If this synthesis of "essential proteins" were to be inhibited by a cycloheximide pulse, the phase would be shifted.

Experiments in the present study are aimed at analyzing the patterns of protein synthesis in the presence and absence of cycloheximide at distinct phases of the cycle (middle of light and dark phases at 20° and 25°). It has been shown that at least one polypeptide (polypeptide x) exhibits an extraordinary behaviour. In the presence of cycloheximide, it appears in the chloroplast membrane in the light phase at 25° and in the dark phase at 20°. The phases of the appearance of polypeptide x in the membrane correlate with the phases at which cycloheximide would phase shift the clock as predicted by the model; i.e. in the presence of cycloheximide polypeptide x appears in the chloroplast membrane at phases where the synthesis of the "essential proteins" should be inhibited according to the model. It seems, therefore, that polypeptide x is not the "essential protein" of the model. However, the extraordinary behaviour of the appearance of polypeptide x in the chloroplast membranes which is mirror-like to the predicted synthesis of the "essential proteins" suggests that there is a close relationship between these two components. For example, one might think that polypeptide x acts as an acceptor for the

"essential proteins". The possible role of polypeptide x in the circadian clock and the interrelationship between the synthesis of polypeptide x and the model will be discussed in the following sections.

2. Quantitative correlation of phase shifting and the biosynthesis of polypeptide x

The experiments reported here show that at least one (polypeptide x) protein is synthesized in the presence of cycloheximide and appears in the chloroplast insoluble fraction during 1600 through 1930 CT at 20° and 0400 through 0730 CT at 25°, but is barely synthesized during 0400 through 0730 at 20° and 1600 through 1930 CT at 25°. Besides the fact that the conditions under which this protein is synthesized in the presence of cycloheximide correlates strikingly with the conditions under which cycloheximide can phase shift the photosynthesis rhythm of Acetabularia, there is a quantitative correlation between the cycloheximide sensitivity and the duration of phase delay. It has been reported that the phase of cycloheximide sensitivity is 4 - 6 hr longer when cells are kept at 20° rather than at 25° (Karakashian and Schweiger, 1976c). From these experiments it is obvious that incorporation into polypeptide x in the presence of cycloheximide is higher at 20° dark phase than at 25° light phase (Figs. 2b, 2c, 3d, 3f, 4d, 4f and Tab. 1), i.e. the higher the incorporation into polypeptide x, the longer is the phase delay.

According to the phase response curves which summarize the effect of cycloheximide at 20° and 25°, the phase delay resulting from cycloheximide treatment at 20° is longer than at 25°, the ratio of durations being 1.4 (13 hr at 20° versus 9 hr at 25°) (Karakashian and Schweiger, 1976 c). Comparisons

of the incorporation into polypeptide x in the presence of cycloheximide at 20° dark phase with that at 25° light phase as presented in this study give values of 1.3 (Figs. 2b and 2c); 1.1 (Figs. 3d and 3f); 1.3 (Figs. 4d and 4f); 1.1 (Tab. 1) and an average value of 1.2. The quantitative correlation between the temperature effect on phase shifting and on the synthesis of polypeptide x is striking enough, since the rate of protein synthesis should increase with increasing temperature. These correlations therefore might be important in evaluating the role of polypeptide x and maybe similar proteins in the biological clock and support the suggestion that the synthesis of these proteins is responsible for phase shifting the circadian rhythm in Acetabularia or vice versa.

3. Polypeptide x as a component of chloroplast membranes

The fact that polypeptide x is only detected in the EDTA-insoluble chloroplast fraction indicates that the chloroplast membrane may be the center of interest since this fraction has been shown to consist mainly of chloroplast membrane fragments (Apel and Schweiger, 1973). Furthermore, at the end of the 3.5 hr of incubation in the presence of cycloheximide, most of the cells were so susceptible to the drug that they appeared yellowish when compared to control cells which remained green; clumps of green chloroplasts were observed against a yellowish background (unpublished results), indicating a possible membrane effect.

The observation that polypeptide x still remains within the chloroplasts even after extensive EDTA extraction indicates that it may be burried deep in the chloroplast membranes or it is very firmly associated with lipids or chlorophyll like most of the photosystem I and II complexes. The amino acid

composition of polypeptide x might also support the idea that it is part of the chloroplast membranes. The proportion of intermediate amino acid group is rather high and the proportions of hydrophilic and hydrophobic amino acid groups well balanced, as expected for most membrane proteins.

The observation that the effect of cycloheximide on the synthesis of polypeptide x is different at 20° and 25° is also consistent with the idea that polypeptide x is most probably part of the chloroplast membranes. Lipids of artificial and natural membranes are shown to change from fluid to solid state at a critical temperature (Oseroff et al., 1973; Singer, 1974). This phase transition has been demonstrated in various membrane systems using such physical methods as spin labelling, X-ray diffraction analysis and differential thermal analysis (Champman, 1970). The thermal phase transition of lipids may induce conformational changes in proteins closely associated with them. It has been reported that the rate of light-induced H^+ transport in spinach chloroplasts was reduced by lowering the temperature; and the Arrhenius plot applied to the first-order rate constant of the H^+ efflux showed a discontinuity at about 20° (Yamamoto and Nishimura, 1976). A similar break or discontinuity at room temperature in the Arrhenius plot has been reported for the energy transduction process of chloroplast membranes (Kraayenhof and Katan, 1971; Raison, 1973; Shneyour et al., 1973; Graeber and Witt, 1974). The strikingly different effect of cycloheximide on the synthesis of polypeptide x at 20° and 25° could for example be attributed to the fact that polypeptide x is a part of the chloroplast membranes which themselves are rather sensitive to temperature changes and consequently could change the polypeptide x binding and hence regulate polypeptide x biosynthesis by a feedback mechanism.

Membrane models (Njus et al., 1974; Sweeney, 1974) are rather popular and attractive in explaining the circadian clock mechanism. However, only general ideas have been offered. The present study shows for the first time that the synthesis of a polypeptide which is closely associated with the chloroplast membranes or which may even be a component of the chloroplast membranes, is directly linked to the phase shifting effect.

4. Synthesis of polypeptide x

The amount of label which appears in the polypeptide x peak is being interpreted as a reflection of the amount of polypeptide x biosynthesis. However, this is not the only interpretation. It is possible, for example that the labelling of polypeptide x depends upon the processing of polypeptide x or upon the rate of integration of polypeptide x into the membrane. However, since no significant amount of polypeptide x or a potential precursor have been found in the cytosolic, EDTA-soluble chloroplast and cell wall membrane fractions, it is concluded that the labelling of polypeptide x reflects true rates of polypeptide x turnover, probably biosynthesis.

The inhibiting effects of cycloheximide would generally be interpreted to mean that polypeptide x is synthesized on 80S ribosomes. Since polypeptide x is an organellar (chloroplast) protein, the possibilities of polypeptide x synthesis on 80S ribosomes need to be considered.

Cycloheximide in the low range of concentration is a specific 80S ribosome inhibitor (Vazquez, 1974). In previous studies with isolated chloroplasts (Apel and Schweiger, 1973), it has been shown that chloramphenicol, a specific inhibitor of

protein synthesis on 70S ribosomes could inhibit only up to about 60 % of the incorporation of labelled amino acids into the TCA-insoluble fraction, while the effect of the two antibiotics (chloramphenicol and cycloheximide) is additive (up to 90 % of inhibition), the conclusion is that part of the chloroplast proteins is synthesized on 80S ribosomes (Goffeau and Brachet, 1965; Nelles and Parthier, 1969; Hooper, 1972; Apel and Schweiger, 1973; Joy and Ellis, 1975).

Indications are that part of the 80S ribosomes which are found in the chloroplast preparations are so firmly attached to the chloroplasts in Acetabularia that they cannot be removed even by extensive purification steps. Such bound 80S ribosomes may be active in synthesizing specific proteins different from those synthesized by free polyribosomes of the 80S type as was shown in Acetabularia (Apel and Schweiger, 1973) and in free and membrane-bound ribosomes of hepatic cells (Ganoza and Williams, 1969) and pancreatic microsomes (Redman, et al., 1966). Furthermore, it has been suggested that the proteins of membranes are synthesized by membrane-bound 80S ribosomes (Dallner et al., 1966).

Similar attachment of 80S ribosomes to chloroplast membranes has been observed in isolated chloroplasts of Vicia faba (Hachtel, 1976) and in Nicotiana rustica, in which newly synthesized cytoplasmic ribosomes are found to be bound to the chloroplast membranes (Lerbs and Wollgiehn, 1975). Developing chloroplasts (transformed etioplasts) which are observed at a time when protein synthesis is rapidly occurring, appear to have cytoplasmic ribosomes associated with the outer surface of the chloroplast envelope (Stocking and Lin, unpublished) as is evidenced in the electron micrograph of a

developing bean leaf (Falk and Stocking, 1976).

It is, therefore, tempting to postulate that some 80S ribosomes are bound to the chloroplast outer membrane in a periodic manner just as the 70S ribosomes bind to the thylakoid membrane of the chloroplast in Chlamydomonas (Chua et al., 1976). Like the 70S chloroplast ribosomes, it might be that these 80S ribosomes are only active in the synthesis of specific proteins when they are bound to the membrane. If this were so, the maximum amount of polypeptide x would coincide with the maximum amount of bound 80S ribosomes.

The inhibitory/stimulating effects of cycloheximide observed in this study have rendered it difficult to predict whether polypeptide x is synthesized on 70S or 80S ribosomes. Considering only the controls at 20° (Tab. 1), the maximum in the rate of polypeptide x synthesis is in the light phase and the minimum is in the dark phase; however, the minimum phase is the time when cycloheximide has a stimulating effect relative to the controls at this time instead of an inhibitory effect. At 25°, there is less evidence for a rhythm of polypeptide x synthesis in the absence of cycloheximide, although cycloheximide induces a stimulation in the light phase and an inhibition in the dark phase in a similar but opposite fashion to what is seen at 20°. If polypeptide x is in fact synthesized on 80S ribosomes, then this stimulation above the control level needs to be explained.

Such a stimulation could occur if there were a temperature and time dependent effect of cycloheximide on the synthesis of some proteins and not on others (e.g. the sub-units of

the photosystem II complex, which are always inhibited by cycloheximide, Tab. 2.) with a consequent increase in the amino acid pool concentration (Kloppstech and Schweiger, 1974) which in turn increases the rate of synthesis of proteins less sensitive to cycloheximide. Such a differential effect of cycloheximide has been indicated in potato tuber discs (Lamb and Rubery, 1976).

The lack or apparent lack of inhibition of the synthesis of polypeptide x by cycloheximide might also be explained by the presence of a cycloheximide sensitivity factor, which itself undergoes oscillation. A cycloheximide sensitivity factor required for N-acetylphenylalanylpuromycin formation has recently been isolated from yeast (Somasundaran and Skogerson, 1976). In the absence of this factor (a protein), 10^{-3} M cycloheximide (281 $\mu\text{g/ml}$) was required for 50 % inhibition of polyphenylalanine synthesis, while in its presence, 10^{-6} M cycloheximide (0.28 $\mu\text{g/ml}$) gave 50 % inhibition.

For example, at 20° in the light phase, polypeptide x is synthesized at maximum rate and if the cycloheximide sensitivity factor were present in its maximum amount, then polypeptide x biosynthesis would be inhibited in the presence of cycloheximide. However, in the dark phase at 20° , if the cycloheximide sensitivity factor were not present (or only at low level), then the concentration of cycloheximide would not be enough to induce an inhibition of polypeptide x and a stimulating effect would be observed as explained above.

A general case could also exist in which a protein synthesized on 80S ribosomes inhibits polypeptide x biosynthesis. If this inhibitor were also oscillating but out of phase with polypeptide x, then a decrease in the synthesis of the inhibitor

in the presence of cycloheximide would produce an increase in the amount of polypeptide x synthesized. If the stimulation of polypeptide x biosynthesis were greater than the inhibition of polypeptide x biosynthesis by cycloheximide, then an apparent stimulation of polypeptide x biosynthesis by cycloheximide would be observed. This effect can be even further accentuated by an increase amino acid pool as described above.

Judging from the fact that the synthesis of polypeptide x is not inhibited by cycloheximide (dark phase at 20° and light phase at 25°), it may be possible that polypeptide x is not synthesized on 80S ribosomes but on 70S ribosomes, although this would not explain the inhibitory effect of cycloheximide during light phase at 20° and dark phase at 25°. Factors which have been applied above to explain the inhibitory/stimulating effects of cycloheximide on 80S ribosomes might also apply to 70S ribosomes although evidence from the literature is lacking.

Another possibility is that the synthesis of polypeptide x is the result of an interplay between the chloroplast and the cytosol; contributions from both the chloroplast and the cytosol being required for the synthesis of polypeptide x, as in the case of synthesis of the large and small sub-units of ribulose diphosphate carboxylase (E. C. 4.1.1.39) (Ellis, 1975).

5. Properties of purified polypeptide x

A reasonable amount of sufficiently pure polypeptide x has been isolated from the EDTA-insoluble chloroplast membrane fraction of Acetabularia. The purity of the isolated protein has been demonstrated by reelectrophoresis and by amino-terminal analysis which shows that there is only one spot

corresponding to glycine, the amino-terminal amino acid of polypeptide x.

Under uncontrolled conditions, it appears to be possible that glycine, the origin of which cannot be accounted for, may contaminate amino-terminal analysis (Menke, personal communication) in which glycine is apparently not the amino-terminal amino acid. However, in the present study, the sizes of all glycine spots correspond roughly to the quantities of material (both polypeptide x and standard amino acid) applied, therefore, it is concluded that the glycine spot detected in the present study is due to glycine originated from the amino-terminal and not otherwise.

The biochemical properties of the purified polypeptide were further investigated by amino acid composition and peptide mapping. The amino acid composition of the purified polypeptide x exhibits properties very close to those expected for membrane proteins. The amino acid composition of polypeptide x is also quite similar in some respect to other chloroplast membrane components although it is possible to differentiate it from them by its own characteristic properties.

The molecular weights of the different peptide fragments obtained from limited proteolysis were determined for each enzyme and added up to give the molecular weight of polypeptide x. The good agreement in the molecular weight determination from different enzyme treatments and from the analytical SDS electrophoresis of intact polypeptide x indicates that polypeptide x is a true protein of high purity and that the methods of molecular weight determination give a reliable value of 39,000.

Trypsin has been known for catalysing the hydrolysis of only those peptide bonds in which the carbonyl function is donated by either a lysine or an arginine residue. One would, therefore, expect to find 38 peptide fragments after trypsin digestion of polypeptide x (25 for lysine and 13 for arginine). However, only four bands could be detected in the gel. This phenomenon may arise when the greater part of the lysine and arginine residues is clustered in one or very few sites on the primary amino acid sequence and therefore most of the peptide bands are too small to be detained by the gel system. Another possibility would be that the conditions of limited proteolysis in the present study and the conformation of polypeptide x are such that the enzyme could gain access to only a few residues, while the others are sort of hidden or protected from the attack. These explanations would also explain observations in other hydrolytical enzymes.

6. Possible role of polypeptide x in regulating the circadian rhythm

As previously discussed, polypeptide x and perhaps similar proteins are in some way related to the phase shifting of the circadian rhythm in Acetabularia. Exactly what this relationship is and how polypeptide x performs its function within the overall mechanism is still unknown. A comparison of the properties of polypeptide x and the "essential proteins" of the "coupled translation-membrane model" (Schweiger and Schweiger, 1977) is shown (Tab. 5). The differences between polypeptide x and the "essential proteins" do not allow the two proteins to be equated; however, the qualitative and quantitative correlations of the amount of phase shifting and the amount of polypeptide x synthesized in the presence of

Table 5. Comparison of the properties of polypeptide x and the "essential proteins" of the "coupled translation-membrane model" (Schweiger and Schweiger, 1977).

Properties	Polypeptide x	"Essential proteins"
Location of protein	Chloroplast membrane.	Unspecified membrane
Site of synthesis	On 80S ribosomes (based on inhibition of synthesis by cyclo- heximide.)	On 80S ribosomes
Time of normal biosynthesis (as judged by the inhibitory effect of cyclo- heximide)	Dark phase at 25° Light phase at 20°	Light phase at 25° Dark phase at 20°
Time of cyclo- heximide stimulated bio- synthesis	Light phase at 25° Dark phase at 20°	Not included in the model
Rate of loading into membrane	Relatively rapidly (no difference being detected between the instantaneous rate at 20° and 25°)	Relatively slowly (taking about 12 hr longer to load at 25° compared with 20°)
Correlation with phase shifting by cycloheximide	Phase shifting by cycloheximide correlated with the amount of poly- peptide x induced by cycloheximide.	Phase shifting by cycloheximide is due to an inhibition of the synthesis and hence absence of "essential proteins".

cycloheximide argue strongly that the biosynthesis of polypeptide x is involved in the circadian rhythm mechanism.

One possibility is for example, that immediately after the synthesis of polypeptide x, it is incorporated into the chloroplast membranes, thereby changing the properties of the membranes (permeability, ion transport, conductivity, electrical charges, etc.). When the concentration of incorporated polypeptide x has sufficiently perturbed the chloroplast membranes, the function of the "essential proteins" which regulates the clock is disturbed with a resulting phase shift. The polypeptide x could also directly interact with the "essential proteins", thereby bringing about changes in the function of the "essential proteins".

Another possibility is that the functioning of polypeptide x depends upon the interaction with a second protein oscillating out of phase (may be even a protein synthesized on 70S ribosomes). The amount of phase shifting is proportional to the amount of interaction resulting from both proteins being simultaneously present. Under free running conditions, the extent of interaction is at a reference level. Perturbation in polypeptide x synthesis (by cycloheximide, light pulses, etc.) will change the level of interaction higher or lower than the reference level, thus resulting in advance or delay of the rhythm. In this particular case, both polypeptide x and the second protein are "essential proteins".

Although the unusual stimulating effects on protein biosynthesis of cycloheximide render interpretation of the results more difficult, the present studies are in agreement with a general interaction in the circadian rhythm mechanism of protein

synthesis and membrane loading of these proteins. Modulation of protein synthesis, perhaps as a consequence of this loading, is supported by the data (Tab. 1) in which control levels of incorporation at 20° but not at 25° show a rhythmicity in the concentration of polypeptide x. Further studies will be necessary to clarify the differences at 20° and 25°.

7. Future outlook

With the purity of the isolated polypeptide x established, it would be possible to obtain antibodies against this protein. This would make it possible to investigate the biological effect of polypeptide x on the circadian rhythm, the localization of the polypeptide x in the chloroplast membranes, the function of the polypeptide x in photosynthesis and the bioenergetics of the proteins. These studies, including experiments clarifying (a) the stimulating effect of cycloheximide, (b) whether phase shifting is responsible for the synthesis of polypeptide x in the presence of cycloheximide or vice versa, and (c) whether there is a rhythm in the amount of polypeptide x under control conditions in the absence of cycloheximide; would be necessary before conclusions on the role of polypeptide x in the mechanism of circadian rhythm could be drawn.

E. SUMMARY

A temperature and phase dependent effect of cycloheximide on the incorporation of radioactive amino acids into the EDTA-insoluble chloroplast membrane fraction of Acetabularia has been observed. Using this differential effect of cycloheximide, at least one polypeptide (polypeptide x) can be recognized, the synthesis of which in the presence of cycloheximide is strongly inhibited at 20° in the light phase and at 25° in the dark phase, but is stimulated at 20° in the dark phase and at 25° in the light phase.

The time of synthesis of polypeptide x in the presence of cycloheximide coincides with the time of sensitivity towards cycloheximide for phase shifting the circadian rhythm of photosynthesis in Acetabularia. Furthermore, the amount of polypeptide x synthesized seems to correlate with the amount of phase delay, i.e. the greater the amount of synthesis of polypeptide x, the greater the amount of phase delay. This suggests that the phase of the circadian rhythm is shifted by the synthesis of polypeptide x or vice versa.

The molecular weight of the polypeptide x was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and is found to be about 39,000.

Polypeptide x was isolated and purified from the EDTA-insoluble chloroplast membrane fraction by means of preparative gel electrophoresis. The purity of the isolated protein has been confirmed by reelectrophoresis. The homogeneity of the isolated protein was further elucidated by amino-terminal analysis which shows only one spot of dansylated glycine

arising from the amino-terminal.

The amino acid composition of polypeptide x shows that the proportion of the intermediate amino acid group is high while the proportion of the hydrophilic amino acid group is well balanced by that of the hydrophobic amino acid group, a property characteristic of membrane proteins.

The polypeptide x was treated with different proteolytes and the peptide fragments analyzed on SDS gel electrophoresis. Peptide fragments ranging from 1,000 to 30,000 in molecular weight were obtained. The molecular weight of each peptide fragment was determined and the total of the peptides arising from each enzyme added up to about 39,100, in very close agreement with the molecular weight of intact polypeptide x as determined by SDS gel electrophoresis, and the molecular weight of 39,700 as determined from the amino acid analysis.

The amino-terminal of polypeptide x was investigated by dansylating the protein and analyzing the dansylated products on polyamide plates coated on both sides. Comparison with dansylated standard amino acids which were co-chromatographed on the same polyamide plate leads to the unambiguous assignment of the amino-terminal to be glycine.

Properties of polypeptide x are in general agreement with models which propose an interaction of the circadian rhythm mechanism of protein synthesis and membrane loading of these proteins.

F. ABBREVIATIONS

EDTA	(ethylenedinitrilo)tetraacetic acid disodium salt
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
SDS	sodium dodecyl sulfate
PPO	2,5-diphenyloxazole
dansyl	5-dimethylaminonaphthalene-1-sulfonyl
CT	circadian time
MW	molecular weight
MES	2-(N-morpholino)ethane sulfonic acid
FSB	final sample buffer
CX	cycloheximide
nmol	nanomoles

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I. CURRICULUM VITAE

I was born on 3. 12. 1946 as the 4th son to the family of high school physics teacher See Choo Leong and primary school teacher Soon Yee Foong in Ipoh, Malaysia.

After six years primary school and three years lower secondary school education (Chinese medium of instruction) at Yuk Choy High School, Ipoh, where I passed the Lower Certificate of Education, I continued my higher school education (English medium of instruction) at St. Michael's Institution, Ipoh, and obtained division one Federation of Malaya Certificate of Education and Higher School Certificate in 1963 and 1965.

At the end of 1965, I was awarded the Japanese Government (Mombusho) Scholarship, with which I completed the Japanese Language Course at Chiba University, Chiba, Japan in 1967, and was awarded the degrees of Bachelor of Engineering (Industrial Chemistry) and Master of Engineering (Industrial Chemistry) at Kyoto University, Kyoto, Japan in 1971 and 1973 respectively. In the summer of 1970, I was sent to the Max-Planck-Institut für Zellbiologie, Wilhelmshaven by the IAESTE, Japan for two months of practical training.

After finishing my study at Kyoto University in 1973, I joined Abteilung Schweiger, Max-Planck-Institut für Zellbiologie, Wilhelmshaven as a post graduate student and remained there until now.

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